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Biochemical Characterization of Human Thy 1

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BIOCHEMICAL CHARACTERIZATION OF HUMAN THY 1

by

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ABSTRACT

LYNDA F. BONEWALD. Biochemical Characterization of Human Thy 1. (Under the direction of Dr. An-Chuan Wang.)

The present studies were undertaken in order to a) verify the presence of a human Thy 1 antigen, b) demonstrate its similarity to murine Thy 1, and c) biochemically characterize the molecule.

Previously, a 25,000 molecular weight molecule had been isolated from a human T lymphoblastoid cell line, Molt 3, using isolation procedures most frequently used for the isolation of rodent Thy 1. The human Thy 1 (p25) was demonstrated to be biochemically similar to murine Thy 1.2 by molecular weight and ability to bind Lens Culinaris lentil lectin. Recently, we confirmed similarity between the murine and the human molecule by peptide maps and amino acid compositions. In addition, a strong cross-reactivity using an anti-p25 antiserum was established with human IgG subclasses. The antiserum did not react with human IgM or IgA, nor with primate immunoglobulins or a battery of other antigens. Through a variety of immunoabsorption experiments, through the use of various digestions, and known amino acid sequences, the shared areas of homology were tentatively determined to be in the intact disulfide bonds of the first and third domains of human IgG and the 9-112 disulfide bond of Thy 1.

A form of the p25 antigen noted and partially characterized was the p40 antigen, the dimerized form of the p25. The p25 and p40 are essentially identical in several aspects, such as amino acid compositions, peptide maps, carbohydrate compositions, reactivity to anti-p25 serum, and in aggregation studies. It was determined that the

detergent sodium dodecyl sulfate tends to convert the p25 molecule into the p40 form through some other chemical means than disulfide bond interchange. The method of isolation and reagents used unequivocally determines observed molecular weight.

Another form detected is the p16 which also reacted with anti-p25 serum, was similar by amino acid composition, slightly different by peptide mapping and definitely contained less carbohydrate than the p25 antigen. The p16 is possibly a cryptic antigen, a breakdown form of the p25 molecule.

A 40,000 MW molecule was isolated from human thymus which binds lentil lectin, reacts with antiserum made to the p25 antigen and possesses an amino acid composition very similar to that of the p25 antigen. Data suggests that the Thy 1 antigen from human thymocytes tends to dimerize more readily than that found on Molt 3 cells and that this antigen is present in considerably smaller amounts on human thymocytes as compared with Molt 3 cells.

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LIST OF ABBREVIATIONS

p25	a 25,000 molecular weight molecule isolated from Molt 3, a lymphoblastoid T cell line
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
MW	molecular weight
DOC	deoxycholate
A.A.	amino acid
SWM	sperm whale myoglobin
Ig	immunoglobulin
DPBS	Dulbecco's phosphate buffered saline
BSA	bovine serum albumin
ABTS	2'2'-azino-di-3-ethyl-benzthiozolin-sulfonate
CnBr	cyanogen bromide
PBS	phosphate buffered saline
ELISA	enzyme linked immunosorbant assay
p40	a 40,000 molecular weight molecule shown to be the dimerized form of p25 antigen
p16	a 16,000 molecular weight molecule thought to be a breakdown product of the p25 antigen
HPLC	high pressure liquid chromatography
TCA	trichloroacetic acid
agg. p25	aggregated p25 of apparent 110,000 molecular weight
agg. p40	aggregated p40 of apparent 110,000 molecular weight
LMW	low molecular weight
RPMI	Roswell Park Memorial Institute media
TMS	Tri-methyl silylating agent
GC	gas chromatograph

CHAPTER 1

THE THY 1 MOLECULE

INTRODUCTION

The Thy 1 molecule was originally described as an antigen on mouse thymus and brain cells by Reif and Allen in 1964 (1). In the mouse there exists two serologically identified alloantigens, Thy 1.1 (AKR) and Thy 1.2 (C3B) (1). The antigen has proved to be a useful cell surface marker for thymocytes and mature T lymphocytes in mice (2). The number of antigenic sites per thymocyte was determined to be greater than 400,000 for Thy 1.2 (3) and about 600,000 for Thy 1.1 (4). The antigenic activity of brain cells is approximately equal to thymocytes on a packed tissue basis (1).

Thy 1 was one of the first cell surface differentiation antigens to be identified on lymphocytes. The expression of Thy 1 varies during different stages of differentiation e.g. in mice it is present on thymocytes and peripheral T cells but not on bone marrow (2) or haemopoietic cells (5). Such is not the case in the rat. The amount of antigen on rat and mouse thymocytes and brain is similar as is the brain developmental pattern, but most peripheral rat T cells lack the antigen. In rats Thy 1 appears earlier in development than in mice. It is present on the pluripotent haempoietic stem cell, early lymphoid, (both T and B) and non-lymphoid (megakaryocytes, mast cells, and some eosinophils) progeny (4,6-8). Rat Thy 1 possesses the Thy 1.1 antigenic determinant but not the Thy 1.2 determinant (9). Xenogeneic (rabbit) antiserum has been raised which detects a rat specific determinant and rat-mouse cross reacting determinants (10). Antigenic sites on the Thy 1 molecule can be divided into determinants specific for the Thy 1 of the immunizing species and determinants which cross-

react between species (11-13). Thy 1 is present extracellularly; in association with collagen based connective tissue in bone blood vessels in rat lymphoid organs (14). In rodents, fibroblasts (15), some epidermal cells (16), breast cells (17), and muscle cells (18) also display the Thy 1 antigen.

Identity of tissue distribution is usually taken as a strong indication of homology, but this is not the case for Thy 1. Thy 1 is found in similar concentration in canine brain when compared to rodent brain, but there are considerably smaller amounts of canine Thy 1 in thymus, lymph node, spleen, and bone marrow. Surprisingly, the antigen was detected in fairly large amounts in canine kidney which has not been seen in the murine system (13). Unlike the mouse, Thy 1 is not a marker for T cells in the Syrian hamster but is also expressed on the majority of resting, peripheral B cells (19). In the chicken a 25,000 molecular weight molecule has been isolated from brain and is assumed to be the Thy 1 molecule but cannot be detected on chicken thymus (20).

SEROLOGY AND LOCATION OF THE THY 1 MOLECULE IN THE HUMAN

Thy 1 is expressed in large quantities in the grey matter of the cortex and basal ganglia of human brain and lower quantities in white matter of cerebrum and peripheral nerves (21). Little controversy, if any, exists concerning Thy 1 in human brain, however, contradictions exist in the literature concerning the location of Thy 1 in human thymus (22-24).

Originally Thy 1 was not detected at all in human thymocytes (13,25), although easily detected on human brain in amounts similar to murine Thy 1 using cross-reacting antibodies to rat Thy 1. None was detected on various organs and tissues at that time. McKenzie and Fabre (26) detected human Thy 1 only in discrete areas of the thymus, spleen, and lymph nodes but not on peripheral blood lymphocytes using an antiserum prepared against a human brain Thy 1 antigen. Cross-reacting determinants were discovered on human fibroblasts and human brain by the use of a rabbit anti-rat Thy 1 and by monoclonal antibodies to human brain Thy 1 which also reacted with human fibroblasts (27).

In contrast, Balch and Ades (28) using an antiserum prepared against a 25,000 molecular weight antigen isolated from Molt 3 cells, noted that the p25 antigen was preferentially expressed on peripheral T lymphocytes and not on brain tissue. They also used an antiserum which recognized a 25,000 MW membrane antigen, that was prepared against monkey thymocytes, to localize the antigen on normal human T lymphocytes and human T lymphoblastoid cells (29).

Sagi and Tanigaki (30) isolated and prepared a rabbit antiserum to a glycoprotein from a cultured leukemic T cell, Molt 4. This antiserum reacted with a Thy 1-like glycoprotein isolated from a T leukemic cell, SKW-3. Reactivity to their antiserum was widely distributed in all human organs and tissues but predominated in brain and testis. Thymus, spleen, and peripheral blood leukocyte preparations were positive, with heart, kidney, liver, and skeletal muscle displaying less antigen.

Ritter et al. (31) found human Thy 1 present in 0.1-0.5% bone marrow cels and 0.2-10% thymus. They found that Thy 1 is confined to early stages of T and B lymphocyte development but absent from all myeloid cells. Using a rabbit antiserum to rat brain Thy 1 and monoclonal antibodies to human brain Thy 1 they also found that all Thy 1 positive T cells are situated in the outer thymic cortex.

At this time one can only assume that the controversy arising concerning the distribution of Thy 1 in the human is due to the methods and materials used for the production of antisera and due to the sensitivity of the techniques employed. For example, Ritter et al. (31) noted that the FACS (Fluorescein Activated Cell Sorter) gave consistently higher values than did fluorescent microscopy.

MOLECULAR STRUCTURE

There has been considerable disagreement as to the molecular nature of the Thy 1 molecule. Some investigators have found various sizes for the molecule, such as 40,000 MW (32) or 60,000 MW (33) but the recent general consensus has been a molecular weight of approximately 25,000 (34-36). Some investigators have suggested it to be a glycolipid (37,38), while others have suggested it is a glycoprotein (39,40). The present general consensus is that the molecule is indeed a glycoprotein (33,41,42) while some question remains concerning the presence or nature of a lipid moiety (36) or an association with lipid (34).

The murine Thy 1 antigen isolated using deoxycholate as a detergent has a molecular weight according to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of 25,000 (43) as does rat Thy 1 isolated from both brain and thymocytes (33,44). Thy 1 from canine brain and human brain using the same isolation procedure also has a molecular weight of approximately 25,000 (45) as does Thy 1 isolated from human foreskin fibroblasts (26). Thy 1 isolated from human leukemic T cells also seems to have an apparent molecular weight of 25,000 daltons (29,46). However, the correct molecular weight has been determined by sedimentation equilibrium studies (35) and for rat brain Thy 1 it is 17,500 and for rat thymocyte Thy 1 it is 18,700. Part of the molecule is carbohydrate, therefore the charge/mass ratio gives an anomalous weight of 25,000 by SDS-PAGE. The molecular weight of the polypeptide portion of the molecule is 12,500. The Stokes radius of canine (3.0 nm) and human (3.25 nm) brain Thy 1 is similar to that for the rat Thy 1 (45).

The amino acid compositions of the various species of Thy 1 show varying degrees of similarity. The Thy 1 molecule is fairly hydrophilic in its amino acid composition. Published amino acid compositions for various Thy 1 preparations from various tissues and species are listed in Table 1.

Rat brain Thy 1 was sequenced in 1981 (36) and murine brain Thy 1 in 1982 (47). These sequences can be seen in Table 2. Sequence homology with immunoglobulin was found (48). The rat Thy 1 consists of 111 amino acids with two disulfide bonds from Cys 9 to Cys 111 and Cys 19 to Cys 85. It has three N-linked carbohydrate structures at residues Asn 23, 74, and 98. The murine Thy 1.1 possesses an Arg at position 89 while in Thy 1.2 there is a Gln. The murine Thy 1 has 112 residues and is very similar to the rat Thy 1 except for two areas, one at sequence 26-29 where there are four differences and a deletion and one at residues 63-67 where there are five different amino acids.

The carbohydrate component has been found to be 20-30% of the Thy 1 molecule. One group of investigators found marked differences between rat brain and rat thymocyte Thy 1 carbohydrate compositions (34). Galactosamine was found in brain Thy 1 but not in thymocyte Thy 1 in contrast to sialic acid which was found in much greater amounts in thymocyte Thy 1. Given that differences in composition are likely to reflect much larger differences in structure, these investigators suggest that the carbohydrate chains of brain and thymocyte Thy 1 may be completely unrelated. All Thy 1 isolated from a homogeneous lymphoblastoid cell line was demonstrated to bind to lentil lectin (43). A comparison of Thy 1 from lymphoma and from normal thymocytes showed a

heterogeneity of Thy 1 from normal cells, but not on lymphoma cells (49).

Published carbohydrate compositions are shown in Table 3. As can be seen considerable variability exists. This could be due to carbohydrate complexity of Thy 1 molecule and also due to the nature of the carbohydrate assay accuracy which cannot be compared to the accuracy attained by amino acid composition analyses.

Carbohydrate complexity has also been demonstrated by lectin affinity studies and isoelectric focusing (50). Only 50% of rat thymocyte Thy 1 bound to lentil lectin (34) and the same was shown for mouse thymocyte Thy 1 (50) while all brain Thy 1 binds to this lectin. In addition, 25% of mouse thymocyte Thy 1 was shown to bind wheat germ agglutinin while 100% was bound by concanavalin A. Analysis by isoelectric focusing revealed six charge variants, which after isolation and treatment with neuraminidase showed that heterogeneity was due to varying amounts of sialic acid (50). Another group of investigators found that Thy 1 isolated from immature mouse thymocytes possessed less sialic acid than that isolated from peripheral or mature T lymphocytes (51). Therefore one can assume a considerable degree of heterogeneity of the carbohydrate portion of the various Thy 1 molecules.

Next to the question of function, the second most often posed question is -- How is the Thy 1 molecule anchored in the cell membrane? Is there lipid association or is lipid covalently linked to the molecule? There is no hydrophobic stretch of amino acids in the sequence of the molecule to form a tail piece or section to extend into

or through the lipid bilayer of the cell membrane. Yet, mouse thymocyte Thy 1 can be labelled in membranes by a photo-activated reagent which partitions into lipid (52).

Miller and Esselman (38) have suggested that mouse Thy 1 is a ganglioside using experiments showing that Thy 1 cytotoxicity could be inhibited by various gangliosides. They also suggested it could be a glycolipid (53). In contrast, other investigators have not found Thy 1 antigenic activity in lipid material (54,55). It has since been shown that Thy 1 is not a ganglioside, but a glycoprotein. The molecule can only be isolated in the presence of detergent, usually deoxycholate. Membrane proteins normally associated with lipid bind large amounts of non-ionic or weakly ionic detergents, whereas other proteins do not (56). The Thy 1 glycoprotein forms a micelle and does not bind to the detergent itself (35). In the absence of DOC, the rat Thy 1 forms a large homogeneous complex of approximately 300,000 MW. The complex can only partially be dissociated by 4M guanidinum chloride suggesting that the protein portion is not directly involved in the aggregation (35).

Barclay et al. (34) mentions a peak running in identical manner to stearic acid during carbohydrate analysis of rat brain Thy 1. While sequencing rat brain Thy 1, Campbell et al. (36) could isolate the terminal peptide either in highly aggregated form or after purification in Brig 96. The peptide appeared to have hydrophobic properties, yet did not contain any extended sequence of hydrophobic amino acids. The C-terminal peptide possessed ninhydrin-positive material, glucosamine, and galactosamine. They suggest linkage to lipid and site precedents in the literature such as linkage through sulhydryl and amino groups of

the N-terminal cysteine of Escherichia coli murine-lipoprotein (57) and sulhydryl of the C-terminal cysteine of rhodotorucine A of Rhodosporidiun toruloides (58). But with Thy 1 lipid could not be attached through the sulhydryl of Cys 111 because it is bonded to Cys 9. They suggest that the carboxyl of Cys 111 could be the attachment site for this material. The nature of this hydrophobic moiety remains to be determined.

MOLECULAR BIOLOGY

The gene for Thy 1 is located on chromosome 9 in the mouse (59). The rat Thy 1 gene has been cloned and sequenced (60) and the cDNA sequence suggests a new and different regulatory mechanism for expression of the molecule. The DNA sequence ends early at a position corresponding to amino acid 103, 8 amino acids earlier than predicted from the protein sequence. There is no termination codon, and a presumptive polyadenylation signal which is part of the coding sequence is found 12 nucleotides upstream from the poly(A) tail. One explanation for this observation is that a deletion of the C-terminal coding sequence was retained, however this phenomena has never been described before. The author's explanation is that the sequence at nucleic acid positions 293-298 served as a polyadenylation signal *in vivo* and position 310 which is 12 nucleotides downstream served as the polyadenylation site. Differential poly(A) site selection may control the expression of the C-terminal coding segment.

Levels of Thy-1 expression change dramatically during cell differentiation. Crude thymus extract has been shown to cause mouse Thy negative bone marrow and spleen cells to become Thy 1 positive (61). Moriuchi *et al.* (60) suggest that thymic hormones may induce the genetic mechanism necessary for the expression of Thy 1. They also found nucleotide sequence homology between Thy 1 cDNA and the mouse light chain variable region to be 36%. They propose that the Thy 1 gene may have inherited a remnant of a mechanism involved in gene expression, namely differential polyadenylation such as seen with membrane and secreted IgM.

FUNCTION OF THE THY 1 MOLECULE

The Thy 1 molecule is a prime example of a fairly well-characterized molecule in search of a function. Its function must be fairly consistent in brain since a variety of species possess large amounts of brain Thy 1 (13). Although originally used as a T cell marker in mice the antigen is not equally distributed in thymus or T cells across species, therefore function may be different from that in brain.

Thy 1 cannot be detected in the brain of newborn mice until seven days postnatally (62). The antigen increases to adult levels by day 23 (42). During this time when the bulk of neural development occurs, an increase in number of synaptic connections closely parallels the increase in antigenic expression. The antigen appears enriched in synaptosomal fractions (63). In brain reaggregate cultures, the presence of Thy 1 closely correlates with the appearance of synapses (64,65). Therefore, in the brain Thy 1 has been thought to be exclusively associated with synapses (66). But antibody to Thy 1 injected into rat hypothalmus selectively inhibits carbamyl choline induced drinking (67). This suggests that antibody reaction with Thy 1 results in blockade of cholinergic function. These experiments indicated that Thy 1 has no association with alpha-adrenergic function.

Barclay et al. (34) suggests that function may be mediated by carbohydrate structures since amino acid compositions between brain and thymocyte Thy 1 were very similar, whereas carbohydrate compositions were not. The polypeptide chain may anchor the molecule in the membrane and therefore provide a backbone for the display of different

carbohydrate ligands. These ligands may be recognition sites for glycosyl transferases or lectins (68). Hoessli et al. (51) also suggests that carbohydrate functions in cell-cell communications since T lymphocyte differentiation is accompanied by an increase in sialic acid content of murine Thy 1. Neuraminidase-treated peripheral lymphocytes do not home to lymphoid organs when injected intravenously into syngeneic hosts but are quickly trapped by the liver (69). Thy 1 was also considered to have a role in cell-cell adhesion in studies of mammary tumor cells in culture (70). Specific interaction of anti-Thy 1 sera with Thy 1 antigen redirected the differentiation program of these cells.

Thy 1 may function in mediating selective adhesion of lymphocytes to extracellular substrates during recirculation. The antigen was found to be associated with collagen-based connective tissue around blood vessels in rat lymphoid organs (14). Thy 1 may not only be involved in selective adhesion and migration of not just lymphocytes in circulation, but also of other Thy 1 negative cell types. Ritter et al. (14) suggested a reciprocal relationship between Thy 1 on cells and on connective tissue. For example, in the rat lymph node and spleen, the Thy 1 positive connective tissue is surrounded by Thy 1 negative lymphocytes (7). In the mouse, the vascular basement membranes are Thy 1 negative, while recirculating T cells are positive.

Springer et al. (71) considers Thy 1 a "jumping" differentiation antigen in contrast to a "lineage" antigen which are expressed in an ontogenetically or functionally orderly fashion. "Jumping" antigens will occur on distantly related tissues while more closely related

tissues will not have the antigen. Therefore they have different functional properties from lineage antigens. Carbohydrate appears to be a prominent structural component of jumping antigens. Another striking property of jumping antigens, such as that of the Forrsman antigen, is that the change of antigen expression correlates with cellular migration to a different tissue. Adhesive properties also change (72). Jumping antigens might have similar functions in very different tissues.

Thy 1 has a direct role not only in normal, but also in neoplastic T cell growth control. Support for this is shown by experiments using anti-Thy 1 antibodies to block Con A induced T cell mitogenesis (73) and blockage of T lymphoma cell proliferation (74). Thy 1 can be found on leukemic and cancerous cells. Human melanocytes are derived from neural crest cells on which Thy 1 is expressed. Normal melanocytes do not express the antigen whereas melanocytes in certain naevi and melanoma do (75). These investigators suggested a role for Thy 1 in cell-cell recognition and also stated that detection of the antigen can be a prognostic, biological marker for melanoma. They also found many Thy 1 positive lymphocytes below the melanomas. Similar results were reported for lymphocytes in inflammatory and malignant infiltrates in the skin (76) and lymphocytes infiltrating breast carcinoma (77). They speculated that either the lymphocytes were beginning to express Thy 1 or had acquired such from surrounding medium.

Maino and coworkers (78) used a rabbit anti-mouse brain, which precipitated a 25,000 dalton glycoprotein from Thy 1 positive cells, but not from Thy 1 negative cells, to activate or initiate T lymphocyte

proliferation. Antisera to Thy 1 allo-determinants were neither mitogenic nor inhibitory to anti-Thy 1 induced responses. Their studies suggest a Thy 1 activating determinant, the necessity of an accessory cell, and a mobile plasma membrane necessary for interactions of Fc receptors, H-2, Ia, and T200 glycoproteins.

Sequence homology was found between rodent neuronal Thy 1 and immunoglobulin (47). A lack of alpha-helix and possession of similar circular dichroism also suggests that Thy 1 structure resembles an Ig domain. When compared to IgD by circular dichroism both Thy 1 and IgD show a negative peak at 217 nm. The authors suggest that the molecule exists to be recognized (79). Homology with variable domains and beta$_2$-microglobulin suggests that Thy 1 may be like the primordial immunoglobulin domain and therefore places it in the immunoglobulin superfamily. This has led Jensenius and Williams (79) to postulate that the Thy 1 molecule is the T lymphocyte antigen receptor. They suggest that the role of the primordial Ig domain was to function as a ligand for triggering cell-cell interactions by binding to a recognizer, which was not immunoglobulin-like and that Thy 1 may be the present day molecule with this function. In support of this function, a monoclonal antibody prepared against rat T cells recognizes a cross-reactive determinant on Thy 1 and the Vk light chain of the TEPC15 myeloma protein (80). These latter findings may imply a role for the Thy 1 molecule in antigen and/or mitogen recognition or may represent a cross-reaction or no structural or functional significance.

In conclusion, theories concerning the function of Thy 1 include a role in cell differentiation, in cell adhesion, in cell migration, T

lymphocyte recognition, cell activation and therefore cell-cell communication of some form, all of which remains to be proven.

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Table 1. Amino Acid Compositions. Content (mol %)

	Mouse brain[a] Thy-1.2	Mouse[b] Lympho-blastoid Thy-1.1	Rat[c] brain Thy 1	Rat[c] thymocyte Thy 1 L+	Rat[c] thymocyte Thy 1 L-	Human[d] brain Thy 1	Dog[d] brain Thy 1	Human[e] brain Thy 1	Human[e] foreskin fibroblasts Thy 1
Cys[a]	0.8	n.m.	3.1	3.2	3.2	3.4	2.7	4.2	4.0
Asx	11.1	12.0	12.7	12.6	13.0	7.4	7.5	7.9	7.8
Thr	7.2	8.3	7.6	8.6	8.3	8.3	12.1	9.8	9.1
Ser	8.5	9.5	7.4	7.1	7.9	11.4	9.7	10.6	10.3
Glx	10.2	10.6	9.1	9.1	9.1	8.6	9.7	9.9	11.9
Pro	4.3	4.1	3.6	3.8	3.0	2.2	4.7	4.1	4.8
Gly	5.4	5.7	6.0	4.9	5.0	6.1	5.2	4.9	5.3
Ala	4.9	4.6	3.3	2.9	3.2	4.7	2.2	3.9	3.3
Val	7.3	7.8	7.3	7.4	7.4	9.7	6.0	6.9	6.3
Met	1.8	1.5	1.1	0.9	0.8	1.3	1.7	0.9	0.9
Ile	6.7	3.9	3.9	4.1	4.0	1.9	3.2	2.0	1.9
Leu	8.9	9.4	10.4	11.2	11.1	8.6	10.2	9.8	9.1
Tyr	3.5	3.5	2.0	2.0	2.0	6.9	4.5	4.4	4.6
Phe	3.2	3.2	4.0	3.7	3.8	3.8	2.7	3.3	3.0
His	4.0	3.7	4.1	3.8	4.0	4.0	2.8	4.6	4.0
Lys	6.0	6.6	6.9	7.2	7.0	7.7	6.9	7.2	7.2
Arg	6.2	5.8	7.5	7.5	7.2	4.8	6.9	5.6	6.5

[a]Data from McClain et al (41).
[b]Data from Zwerner et al (42).
[c]Data from Barclay et al (34).
[d]Data from McKenzie et al (45).
[e]Data from Cotmore et al (27).

Table 2. Amino Acid Sequence of Rat and Mouse Thy 1

													10							
Rat Thy 1	PCA	-	ARG	-	VAL	-	ILE	-	SER	-	LEU	-	THR	-	ALA	-	CYS	-	LEU	-
Mouse Thy 1.1			LYS				THR													
VAL	-	ASN	-	GLN	-	ASN	-	LEU	-											
					20														30	
ARG	-	LEU	-	ASP	-	CYS	-	ARG	-	HIS	-	GLU	-	ASN	-	ASN	-	THR	-	ASN
																		LYS	-	ASP
LEU	-	PRO	-		-	SER	-													
ILE	-	GLN	-	HIS	-															
													40							
GLU	-	PHE	-	SER	-	LEU	-	THR	-	ARG	-	GLU	-	LYS	-	LYS	-	LYS	-	HIS
																ARG				
VAL	-	LEU	-	SER	-	GLY	-	THR	-	LEU	-									
50																		60		
GLY	-	VAL	-	PRO	-	GLU	-	HIS	-	THR	-	TYR	-	ARG	-	SER	-	ARG	-	VAL
		ILE																		
ASN	-	LEU	-	PHE	-	SER	-	ASP	-	ARG	-									
THR						SER	-	ASN	-	GLN	-	PRO	-							
					70														80	
PHE	-	ILE	-	LYS	-	VAL	-	LEU	-	THR	-	LEU	-	ALA	-	ASN	-	PHE	-	THR
TYR	-																			
THR	-	THR	-	LYS	-	ASP	-	GLU	-	GLY	-	ASP	-							
													90							100
TYR	-	MET	-	CYS	-	GLU	-	LEU	-	ARG*	-	VAL	-	SER	-	GLY	-	GLN	-	ASN
		PHE								GLN								ALA		
PRO	-	THR	-	SER	-	SER	-	ASN	-	LYS	-									
		MET																		
																		110		
THR	-	ILE	-	ASN	-	VAL	-	ILE	-	ARG	-	ASP	-	LYS	-	LEU	-	VAL	-	LYS
SER				SER				TYR												
CYS																				

*For Mouse Thy 1.2 Position 89 is a GLN.

Table 3. Carbohydrate Compositions. Carbohydrate Residues/per Molecule

	Mouse brain[a] Thy-1.2	Mouse Lympho-blastoid[b] Thy-1.1	Rat brain[c] Thy 1	Rat thymocyte[c]		Human[d] brain	Canine[d] brain
				Thy 1L+	Thy 1L-		
Fuc	1.5	1.8	1.8	1.0	0.9	3.9	4.0
Man	8.8	8.7	11.9	10.6	9.4	13.2	14.2
Gal	4.0	6.3	1.8	5.5	6.9	5.6	4.3
Glu	0.0	0.0	0.6	1.3	1.1		
N-Ac-GluNH_2	4.4	8.6	6.3	7.0	8.3	8.0	10.7
N-Ac-GalNH_2	0.9	0.0	1.0	0.0	0.0	1.8	1.2
NANA	0.4	3.5	0.2	1.8	2.2	1.3	2.7
Total	20.0	23.9	29.0	32.0	35.0	36.0	38.0

[a]Data from McClain et al (41).

[b]Data from Zwerner et al (42).

[c]Data from Barclay et al (34).

[d]Data from McKenzie et al (45).

CHAPTER 2

MOLECULAR COMPARISON OF MOUSE THY-1 AND ITS HUMAN HOMOLOGUE

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ABSTRACT

Physicochemical techniques were used to verify previous immunochemical studies showing homology of the human Thy-1 (formerly p25) antigen and the murine Thy-1 or antigen. Peptide maps and amino acid compositions showed close similarity between these two proteins; however, they were not identical. These data confirm that the p25 antigen is the human homologue of mouse Thy-1.

INTRODUCTION

Few human lymphocyte antigens have been characterized biochemically, other than determinations of their molecular weights. Characterization of cell surface antigens and receptors has been difficult due to the low density of membrane antigens and the large number of cells required for extraction of sufficient amounts of the desired molecules. Differentiation antigens from rats and mice, however, have been isolated in milligram quantities by extraction from tissues or organs (1,2). Using similar techniques, we previously located a T cell antigen with a molecular weight (M.W.) of 25,000 from a human lymphoblastoid T cell line (3). We have now isolated a sufficient quantity of this antigen to determine its peptide map and amino acid (a.a.) composition, and we have compared some biochemical properties of the human p25 and murine Thy-1 antigens. Our results support the conclusion based on previous immunological comparisons that the p25 antigen is the human homologue of murine Thy-1.

MATERIALS AND METHODS

Analytical sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli et al. (4) using 5-20% gradient gels on a Hoefer electrophoresis apparatus. The gels were stained with Coomassie blue. Molecular weight (M.W.) was estimated by comparison with a M.W. marker kit (Pharmacia, LMW). Molt-3 cells from large scale cell cultures (5) were grown in suspension with 15% heat-inactivated fetal calf serum, and isolation of the p25 antigen was performed as described previously (3) with the following modifications. The packed cells were suspended in a pH 7.4 lysing medium of 0.01 M Tris-HCl, 0.015 M NaCl containing 0.01% egg white trypsin inhibitor (Sigma), 0.01 M phenylmethylsulfonylflouride (CalBichem) and 0.0025% DNase (CalBiochem). The fraction eluted from a Sepharose 4B Lens culinaris column (Vector) was concentrated by air dialysis. An Ultrogel AcA-54 column was used, the desired fractions were concentrated by air dialysis, and preparative SDS-PAGE was performed. Approximately 0.5 ml of concentrated eluent was layered on a gel 0.75 mm thick. The sides of the gel were cut off and stained with Coomassie blue to locate the 25,000 M.W. band, which was sliced out, homogenized, and extracted with 0.5% deoxycholate (DOC), 10 mM Tris buffer. The acrylamide was removed from the buffer by high-speed centrifugation (4000 xG) and the purified protein supernatant was concentrated by air dialysis to 1.19 mg/ml. We isolated 0.14 mg of the p25 antigen per ml of packed cells. This isolated antigen was reactive with antisera raised against another preparation of p25 antigen (3).

This preparation of human p25 antigen (0.5 ml) and purified mouse Thy-1.2 (5) (0.45 mg at 1 mg/ml) were analyzed for a.a. composition by standard procedures using a Durrum D-500 automatic amino acid analyzer. Approximately 100 ug of protein was hydrolyzed in 6 N HCl, 0.1% phenol at 110°C under vacuum for 18 hr. For the human p25 antigen additional lyophilization and filtering were performed in order to remove discoloration and particles. This discoloration was assumed to be due the presence of DOC.

Sperm whale myoglobin (SWM) was used as a control and subjected to the same procedures as the Thy-1 and p25 antigens. The corrected a.a. composition was calculated as follows. For each a.a. the known number of a.a. was divided by the known number of residues for SWM, and the resulting figure was divided by the percent composition of the a.a. determined in SWM from our analysis. This factor was used to correct the content of each a.a. for the Thy-1 and p25 antigen by multiplying the observed percent composition to give a corrected percent composition as follows:

$$\text{correction factor} = \frac{(\text{known no. of a.a. in SWM}) \,/\, \text{known no. residues in SWM})}{(\text{observed \% composition in SWM})}$$

and

corrected % composition = (observed % composition) x (correction factor).

A minimum of three runs were used to calculate the compositions of the Thy-1 and p25 antigens.

Tryptic digests were performed on each sample (100-200 ug) in 0.05 M NH_4HCO_3 buffer, pH 7.8, for 4 hr at 47°C with an enzyme:substrate ratio of 1:50. The digest was applied to a 0.1-mm micro-crystalline cellulose CEL 400-10 thin-layer plate (Brinkmann Instruments). First, ascending chromatography was performed using n-butyl alcohol:glacial acetic acid:pyridine:water (15:6:12:10) solvent. Buffer for electrophoresis in the second dimension consisted of glacial acetic acid:pyridine:acetone:water (2:1:8:40) at pH 4.4. Constant current was applied at 300 V for 105 min. After drying, the plate was sprayed with a cadmium-ninhydrin solution prepared by adding a 1% ninhydrin solution consisting of 80% acetone and 20% glacial acetic acid to a 10% solution of cadmium acetate at a ratio of 20:1.

An ethanol precipitation was performed on the remaining 150 ug of material. Three volumes of 100% ethanol were added to each sample, the sample was incubated at -20°C for 48 hr and centrifuged at 2,000 rpm for 15 min, the supernatant was decanted, and the precipitate was redissolved in water. During the ethanol precipitation, the human glycoprotein tended to redissolve when brought to room temperature. Therefore, centrifugation was done at 4°C and the supernatant was immediately decanted. An additional composition and peptide map were obtained using this material.

RESULTS

By SDS-PAGE, human Thy-1 (p25) and mouse Thy-1.2 migrated with the same R_f value. This SDS-PAGE analysis indicated a M.W. of 25,000 for both antigens. In Table 1, a.a. analyses are expressed as the mean number of each residue per 100 residues, excluding tryptophan. The mouse Thy-1 composition was similar to those reported previously (2,5). The human p25 was similar to mouse and rat Thy-1 (1) except in the amounts of glycine and alanine present. The composition of SWM compared favorably with its published composition and was used to derive a correction factor, as described above. This rarely resulted in a correction difference of more than 10%. Hydrolysis of the 0.5% DOC-Tris eluting buffer showed no interference with a.a. resolution.

In order to test whether the high content of glycine and alanine in the p25 antigen (Table 1) was real or an artifact, we performed an ethanol precipitation in order to remove any contamination due to eluting buffers. The glycine and alanine contents remained constant.

The peptide maps obtained for p25 and Thy-1.2 were quite similar although not identical (Fig. 1). Several homologous peptides were found. The two spots that migrated farthest in the electrophoretic direction were arginine and lysine as determined from maps of other proteins containing arginines or lysines in juxtaposition, with no intervening trypsin-sensitive amino acids.

DISCUSSION

Previous immunological data have suggested identity or close similarity between the human p25 and mouse Thy-1 antigens (3). They have similar M.W., they are glycoproteins, and they share identical or cross-reactive antigenic determinants. Our comparison of the a.a. compositions and peptide maps of the two molecules (Table 1 and Fig. 1) indicates that they are closely related molecular species. Differences observed in the migration patterns of the p25 and murine Thy-1 antigen in the chromatography dimension could possibly be due to differences in carbohydrate moieties and not necessarily peptide differences. At present little is known concerning the carbohydrate composition of human Thy-1 antigen, whereas a large amount of data is available concerning the murine and rat Thy-1 antigens (2,5). For example, the carbohydrate composition of murine Thy-1 is about 20% of its M.W. and for the rat this figure is 30% (1). Barclay et al. (1) found a M.W. of 25,000 for rat Thy-1 by SDS-PAGE, but by sedimentation equilibrium the M.W. of rat brain Thy-1 was 17,500 and that of rat thymocyte Thy-1 18,700. Thus they calculated the polypeptide portion to be approximately 12,500 daltons. We assume that the carbohydrate portion of the human antigen is similar to the murine and rat antigens since similarity was observed by peptide mapping. The M.W. of human Thy-1 could be appreciably lower than 25,000. Quantitation of the carbohydrate moiety and its effect on the peptide mapping of these antigens is underway.

Subtle differences were observed not only in the migration patterns of the peptide maps but also in a.a. composition. These minor differences could be due to (a) our isolation procedure, (b) the fact that our antigen was derived from a lymphoblastoid cell line, or (c) differences in cell function. Our technique of isolating large quantities of the p25 antigen by gel and lectin affinity chromatography could yield an antigen different from that retrieved by immunoprecipitation with specific antisera. For example, Barclay et al. (1) isolated a Thy-1 antigen that bound to a lectin column and one that did not; both reacted with anti-Thy-1 serum. They varied only slightly in amino acid composition, with greater differences in carbohydrate composition. Comparisons between lectin-purified p25 and immunoprecipitated p25 are now in progress in our laboratory.

We assume that the antigen isolated from the lymphoblastoid cell line Molt-3 is identical to that found on normal lymphocytes in spite of an altered genome and expression of new antigens in these cells. Support for this assumption derives from our earlier immunoprecipitation experiments (3). Also, in several cases identical molecules have been isolated from normal tissue and from malignant cell lines, including immunoglobulins, rosette receptors, and antigens such as $beta_2$-microglobulin (6), HLA alloantigens (7) and, recently, mouse Thy-1.1 (8).

The functional role of the murine and human Thy-1 antigens is unclear at present. Conservation of structure of Thy-1 antigen between species supports the theory of a necessary specific function. In the

mouse this antigen is found on thymocytes, mature T cells, and brain cells (1). In the rat, brain and bone marrow cells are positive for Thy-1 whereas peripheral T lymphocytes are negative (1). In humans, the p25 antigen seems to be preferentially expressed on peripheral T lymphocytes and not on brain tissue (9). Maino et al. (10) have suggested that the murine Thy-1 antigen is involved in T cell activation and proliferation, and McClain et al. (2) have suggested that the Thy-1 antigens establish synaptic connections which are conserved in a variety of species and tissues. Perhaps the subtle differences between Thy-1 antigens reflect differences in function. Correlations between the structure and function of these antigens, as well as information about their evolution, may be revealed by a.a. sequencing. Such studies are now in progress in our laboratory with murine and rat Thy-1 and with the human p25 antigen.

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Table 1. Amino acid compositions of mammalian lymphocyte Thy-1 antigens

Amino acid	Content (mol %) in						
	Human Thy-1		Mouse Thy-1.2		Mouse Thy-1.2[a]	Mouse Thy-1.1[b]	Rat Thy-1.1[c]
ASX	11.5	(10.0)[d]	12.4	(10.8)[d]	13.3	12.0	12.6
THR	6.7	(6.2)	7.9	(7.3)	8.1	8.3	8.6
SER	6.2	(6.0)	8.3	(8.0)	8.5	9.5	7.1
GLX	12.2	(11.7)	10.8	(10.2)	11.3	10.6	9.1
PRO	5.7	(5.5)	4.1	(3.9)	3.7	4.1	3.8
GLY	10.3	(9.9)	5.7	(5.4)	5.1	5.7	4.9
ALA	10.0	(9.6)	5.0	(4.8)	3.9	4.6	4.8
VAL	7.1	(7.9)	6.2	(6.8)	7.8	7.8	7.4
MET	0.9	(0.8)	2.0	(1.7)	1.3	1.5	0.9
ILE	4.4	(5.3)	3.8	(4.6)	3.8	3.9	4.1
LEU	9.2	(8.6)	13.5	(12.4)	10.0	9.4	11.2
TYR	2.3	(2.1)	3.1	(2.8)	3.4	3.5	2.0
PHE	3.7	(3.0)	2.7	(2.2)	3.0	3.2	3.7
HIS	1.4	(1.9)	2.8	(3.8)	3.6	3.7	3.8
LYS	4.3	(4.9)	6.8	(7.7)	7.6	6.6	7.2
ARG	2.7	(2.7)	4.8	(4.9)	6.3	5.8	7.5

[a]From Acton et al. (5).

[b]From Zwerner et al. (8).

[c]From Barclay et al. (1).

[d]Corrected % composition (see Materials and Methods).

Fig. 1. SDS-PAGE analysis of human Thy-1 (p25) antigen (a) and murine Thy 1.2 (c). Low molecular weight markers (b,d) are shown for comparison.

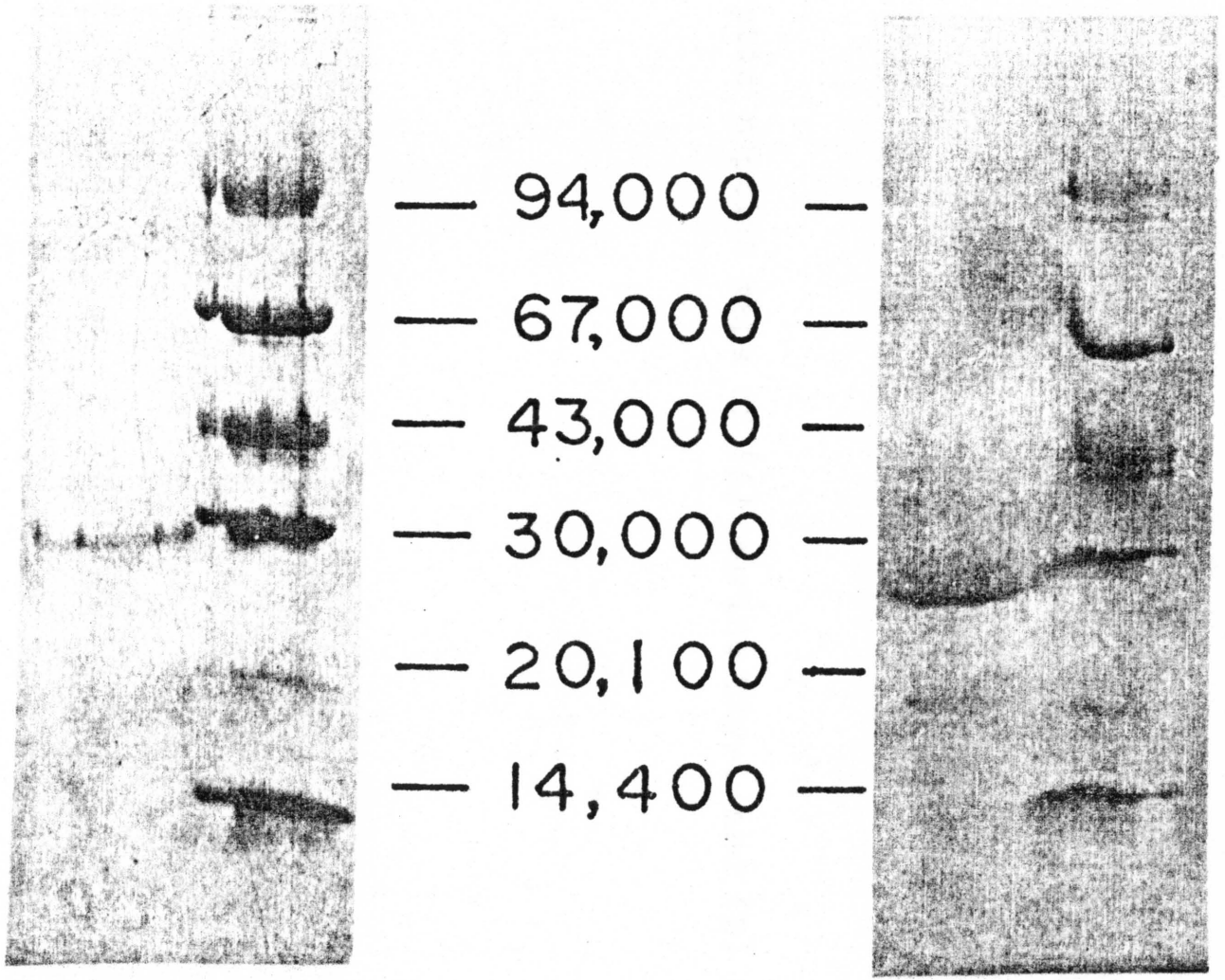
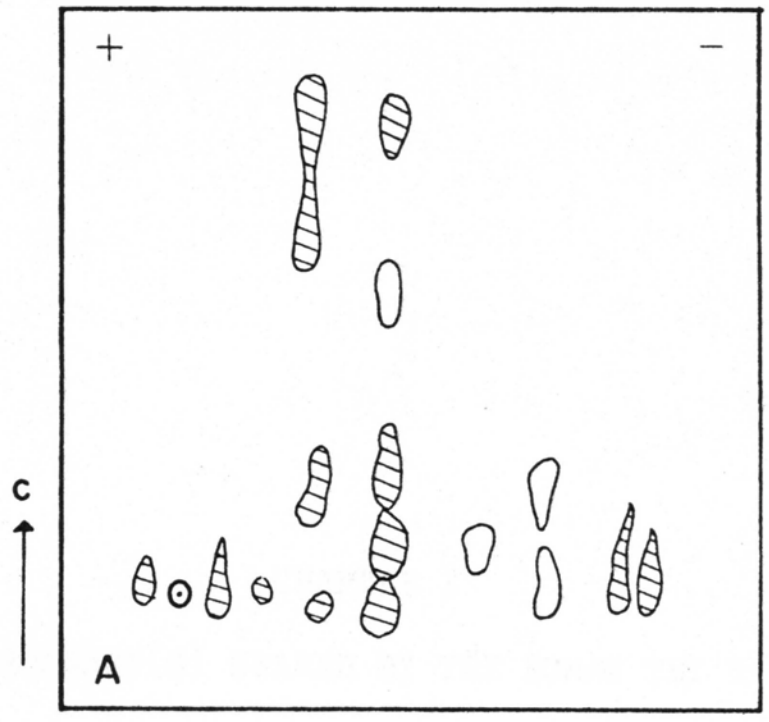
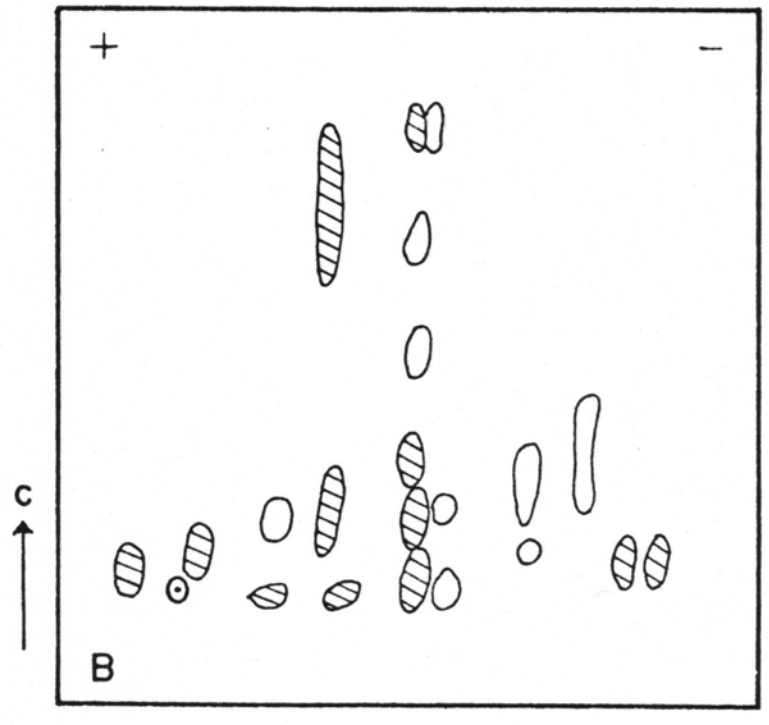

94,000
67,000
43,000
30,000
20,100
14,400
a
b
c
d

Fig. 2. Tryptic peptide maps of (A) murine Thy-1 and (B) human p25 antigens.


+
−
c
A


+
−
c
B

CHAPTER 3

ANTIGENIC DETERMINANT(S) SHARED BY THE HUMAN THY 1 AND HUMAN IgG

(Accepted by Immunobiology, January 1984)

ABSTRACT

A human T cell differentiation antigen (p25) previously described as being the mouse theta equivalent has been examined for shared antigenic determinants with immunoglobulin. A strong cross-reactivity of an antiserum prepared against p25 antigen was established with human IgG subclasses. This antiserum does not react with human IgM or IgA, nor with primate immunoglobulins. The shared determinants appear to be associated with the disulphide-bonded cysteines in the first and third constant domains of the IgG molecule and the 9-112 disulfide bond of Thy 1.

INTRODUCTION

Thymus derived (T) lymphocytes are defined classically by their expression of receptors for sheep red blood cells (1). Other constituents of the T cell membrane include: HLA antigens (2), Fc receptor (4) β_2-microglobulin (3), and numerous antigenic structures associated only with T cells (T cell specific cellular antigens) as defined by either heterologous or monoclonal antibodies (5).

The products of immune responses, specific antibodies, and antigen specific T cell receptors are exquisite examples of recognition units for foreign and self antigenic structure. Whether the Thy-1 antigen is the primordial immunoglobulin domain and possibly the T cell receptor as first proposed by Jensenius and Williams (6) is still enigmatic, although our data render support for this proposal. In this study, we find that an antiserum made to a T cell specific product (7), the human Thy-1 molecule (8), can also react with a B cell specific product, human IgG.

MATERIALS AND METHODS

Antiserum. The antiserum was prepared as described previously (7). Briefly, a 25,000 M.W. fraction of cell membrane was isolated from a human T cell line and injected intramuscularly in complete Freund's adjuvant into a rabbit. The second and third injections were given 14 and 28 days later in incomplete Freund's adjuvant. The antiserum obtained was heat inactivated and used without any adsorptions. Specificity of the antiserum for human T cells is as previously described (7,8).

Cytotoxicity assay. The specificity of the antiserum for different lymphoblastoid cell lines was determined by using the ^{51}Cr release cytotoxicity assay as described previously (9).

Cell lines. The cell lines were graciously donated by Dr. Jean Michel Goust and Dr. Jan Minowada.

ELISA assay. Microtiter plates (Linbro) with 8x12 wells were used. The wells were coated with 100 ul of antigen (1-2 ug/ml). The plates were incubated at 37°C for 2 hr or at 4°C overnight. Plates were washed 3x with Dulbecco's phosphate-buffered saline (DPBS) and blocked with 5% bovine serum albumin (BSA), incubated at 37°C for 30 min or 1% BSA overnight and washed 3x with DPBS containing 0.05% Tween-20. To each well, 100 ul of diluted antibody was added and the plate incubated at 37°C for 2 hr. The plates were then washed 3x with DPBS + Tween. 100 ul of a 1:2000 dilution in 1% BSA of goat anti-rabbit antisera coupled with horse radish peroxidase was added to each well and

incubated for 1 hr at 37°C. The plate was then washed extensively (6x) with DPBS + Tween. Addition of 100 ul/well of 0.03% ABTS (2,2'-azino-di-3-ethyl-benzthiazolin-sulfonate, Boehringer, Mannheim, W. Germany) in 0.1 M Na-citrate, pH 4.0, plus 0.003% H_2O_2 was made. The plate was covered and read between 15 and 30 min. in a Multiscan at 414 nm.

Source of Antigens. Samples used included rat IgG (Miles Laboratory), chicken, cow, and sheep immunoglobulin (Pentex, Inc.), conalbumin (Dr. R.E. Feeney), alpha$_1$-antitrypsin (Dr. Philippe Arnaud), beta$_2$-microglobulin (Ira Rosenshein), and human albumin fraction V. Pooled immunoglobulins from human, gorilla, and rhesus monkey as well as human myeloma IgG, IgA, and Waldenstrom's IgM were isolated from sera by precipitation with sodium sulphate, ion-exchange chromatography using a DE-52 column, and gel filtration chromatography using a Sephadex G-200 or Sephacryl S-300 column as previously described (10). These immunoglobulins were reactive with antiserum made to human immunoglobulins. Human transferrin was isolated by rivanol precipitation, starch-block electrophoresis, and gel filtration chromatography using a Sephadex G-100 column as previously described (11).

Cyanogen bromide (CnBr) coupling to Sepharose 4B. Pooled normal human IgG, G-200 fraction, 150 mg, was coupled to 10 g CnBr activated Sepharose 4B according to the procedure of March et al. (12).

Absorption experiments. The pooled human IgG coupled Sepharose 4B was washed once with 1 M acetic acid, centrifuged, and washed 3x with PBS. Five hundred ul of antiserum was added, the sample was vortexed, and allowed to stand at room temperature for 2 hr. The sample was again

vortexed, centrifuged, and the absorbed antiserum was removed. This absorbed antiserum was used in the ELISA assay to test reactivity to human IgG and the p25 antigen. The IgG coupled Sepharose 4B column was then washed once with PBS and then 2x with 1 M acetic acid. The acetic acid wash was lyophilized and reconstituted to 500 ul with PBS and tested for its reactivity to human IgG.

Molt 4 cells ($1x10^6$) were washed 2x with PBS. Five hundred ul of antiserum was added to the cells and incubated on a shaker for 2 hr at 37°C. The cells were centrifuged and the absorbed antiserum removed and tested in the ELISA assay against human IgG and the p25 antigen. Care was taken to maintain an equivalent volume of antiserum (500 ul) for absorption with the Sepharose 4B coupled with human IgG column, the Molt 4 cells, and in the 1 M acetic acid wash.

Reduction and Alkylation. IgM, IgA, and IgG protein samples were reduced and alkylated and the heavy (H) and light (L) chains separated as described previously (13).

Isolation of IgG Fab and Fc fragments. Papain digestion of human IgG myeloma proteins and isolation and purification of Fab and Fc fragments was performed as described previously (14).

Digestion of Fab fragments. Tryptic digests were performed on each sample in 0.05 M NH_4HCO_3 buffer, pH 7.8, for 4 hr at 37°C with an enzyme:substrate ratio of 1:50. Staph V-8 protease digestion was performed as described in Methods of Enzymology (15). Briefly, protein samples were dissolved in distilled H_2O pH 4.0 at a 10 mg/ml solution

and allowed to equilibrate for 5 min at 37°C. The enzyme was dissolved in 1 M Na acetate pH 4.0 and added to the protein at a ratio of 1:100 and incubated at 37°C for 20 min. The reaction was stopped by the addition of 1% trichloroacetic acid.

RESULTS

Cytotoxicity assays were performed on a number of human and murine T cell lines in order to establish T cell specificity of the antiserum and relative amount of antigen per cell. Results are shown in Table 1. As can be seen from this Table, not all T cells are reactive with this antiserum according to the cytotoxicity assay, however, the antiserum possesses a high reactivity for Balb/c thymocytes.

Determination of specificity of the antiserum was accomplished through use of the ELISA assay. Reactivity to human IgG was determined to be as great as for the human purified p25 antigen (Figs. 1 and 2). The antiserum reacted with three different subclasses of IgG, i.e. IgG_1, IgG_2, and IgG_3 (Fig. 2). There was little or no reactivity to human albumin, conalbumin, $alpha_1$-antitrypsin, transferrin; sheep, chicken, or cow immunoglobulins; to rhesus, baboon, or gorilla immunoglobulin; rat myeloma, human IgM, human IgA, k or λ light chains, reduced and alkylated human IgM heavy chain, IgA heavy chain, or IgG heavy chain. Mild reactivity (approximately 35%) was seen with mouse IgG and rat IgG, but none with mouse IgG_{2b} and IgM (Fig. 3). Both the Fab and Fc fragments from papain digested human IgG reacted with the antiserum (Fig. 4).

In order to determine whether the antiserum was recognizing a determinant shared by both the human Thy-1 antigen and human IgG, absorption with human IgG coupled to Sepharose 4B or with Molt 4 T cells was performed. As can be seen in Fig. 5, the majority of the reactivity of the antiserum to IgG was removed by absorption with IgG while reactivity to IgG was also removed by absorption with Molt 4

cells (Fig. 5c). Partial reactivity to the IgG (Fig. 5d) was recovered from the 1 M acetic acid wash from the IgG coupled Sepharose 4B column. In Figure 6, reactivity to the p25 antigen was removed by absorption with Molt 4 cells and the IgG coupled to Sepharose 4B yet recovered from the 1 M acetic acid wash. Previous studies (7) using immunoabsorption and immunodepletion have shown complete removal of reactivity to the p25 antigen. Furthermore, as can be seen in Fig. 7, reactivity to IgG Fab fragments was removed (1:80) or greatly diminished by trypsin digestion while only moderately diminished by Staph-V8 protease digestion.

DISCUSSION

Thy 1 is a well-characterized membrane antigen with a wide tissue distribution (16). Although it is mainly present in high levels in neuronal cells and T cells, recent studies indicate that it is also detected in immature B cells (17). Recently, new ideas have appeared in the scientific literature purporting a new function for the Thy 1 antigen. Williams & Gagnon (18) have discovered structural and sequence homology between murine Thy 1 and immunoglobulin, especially with the V (variable) region sequence. They suggest that Thy 1 may be like or is the primordial immunoglobulin domain. Jensenius & Williams (6) go so far as to suggest that Thy 1 may replace current concepts of the T cell receptor. According to these investigators, the Thy 1 molecule is the primordial immunoglobulin domain which functions as a ligand which triggers cell-cell interactions by binding to a recognizer and that these two molecules, the ligand and the recognizer, could have evolved to produce the antibody and the histocompatibility systems.

In earlier works, we have shown that the p25 antigen isolated from the human leukemic line, Molt 3 is the human homologue to murine Thy 1 (8). Similar procedures for isolation of both the human and murine Thy-1 antigens were used; they both have identical molecular weights, both bind Lens culinaris lectin and are cross-reactive as shown by immunodepletion and absorption experiments (7). In addition, the p25 antigen and mouse Thy 1.2 have similar though not identical tryptic peptide maps and similar amino acid compositions (8). Therefore, we set out to determine whether the human Thy-1 molecule shares homology with human immunoglobulin.

As can be seen in Figures 2 and 5, the anti-p25 antiserum recognizes a common determinant on the p25 antigen as well as on human IgG. Since both the Fab and Fc fragments reacted with the antiserum (Fig. 4), we began to look for similar sequences in the IgG heavy chain and murine Thy 1 yet, could not be found in human IgM or IgA. The Sequence of Proteins of Immunological Interest Atlas (20) yielded two such positions. One was 123-126 and 238-242 of IgG, but there was not such a sequence in murine Thy-1 (18,19), while the other position was 144-147 and 367-370. However, the amino acids in the latter positions are not exactly in the same sequence as murine Thy 1, thus prompting examination of the disulfide bonds (first and third constand domain) of human IgG. As can be seen in Fig. 8, the sequence C L V-C _ V are conserved in most heavy chain domains. It is the identity of the surrounding amino acids which determine distinctive antigenicity. Reduced and alkylated IgG heavy chain is no longer reactive with the antiserum and on this basis one might predict the sequence of the human Thy 1 disulfide bond and the analogous murine disulfide bond 9-112, as the position which closely resembles or is identical to the disulfide bonds of human IgG. Thus, if the antiserum was recognizing the disulfide bond in human IgG, trypsin should destroy this reactivity since a lysine is located at positions 147 and 370. Also, we predicted that digestion with Staph V-8 protease, which cleaves only at glutamyl residues in the system used (15), would not affect reactivity. These expected results were found with two different IgG Fab fragments as shown in Fig. 7.

The antigen that was injected into the rabbit had been extracted from cell membrane and therefore had antigenic determinants exposed that normally would be buried in the cell membrane. It could be a determinant near the COOH terminal (one of many others), is being recognized by this antisera.

A natural question to be raised, is could antigenicity due to carbohydrate? The Thy-1 molecule has been shown to be 20-30% carbohydrate (9,16,21,22), and have three N-linked carbohydrate chains (18). However, IgG is only 1-3% carbohydrate, has only one carbohydrate moiety at position 237, and Kornfeld found a different sequence of sugars in seven IgG myeloma oligosaccharide chains (23). Other proteins tested have more carbohydrate than IgG: $alpha_1$-antitrypsin is 16%, and IgM is 7-12% with five carbohydrate chains (25). But what answers this question are the data which show 1) that both IgG Fab and Fc fragments react with the antiserum and that the Fab fragment lacks carbohydrate, 2) that trypsin digested Fab fragment looses reactivity to the antiserum, and 3) that reduced and alkylated IgG no longer reacts with the antiserum.

Dales and coworkers have recently discovered monoclonal antibodies with affinity for murine Thy1.1 and Thy1.2 which also bind to actin and vimentin (26). They compared amino acid sequences of Thy1.2, smooth muscle actin and rabbit vimentin and showed suggested sequence homology as we have done with human IgG.

In summary, we have demonstrated that an antiserum made to a human T cell specific product, reacts with a B cell specific product. We

have tried to pinpoint the shared antigenic determinants between human IgG and human Thy 1 through the use of antiserum reactivity, published Thy-1 sequence data, and biochemical modification of the human IgG molecule. Our conclusions of shared antigenicity can only be further substantiated by determining the sequence of the human Thy 1 antigen which is now in progress in our laboratory.

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Table I

^{51}Cr Release Cytotoxicity Assay with Rabbit Anti-Human p25 Antiserum Against T Cells

+	-
BALB/c Thymocytes	HPB-ALL
HPB-MLT	Molt 21
Molt 3	SB
Molt 4	HSB
JM	CEM-pellet
CEM band	W.M. (woolly monkey T cell)

Fig. 1. ELISA assay showing reactivity of the p25 antigen with the anti-p25 antisera.

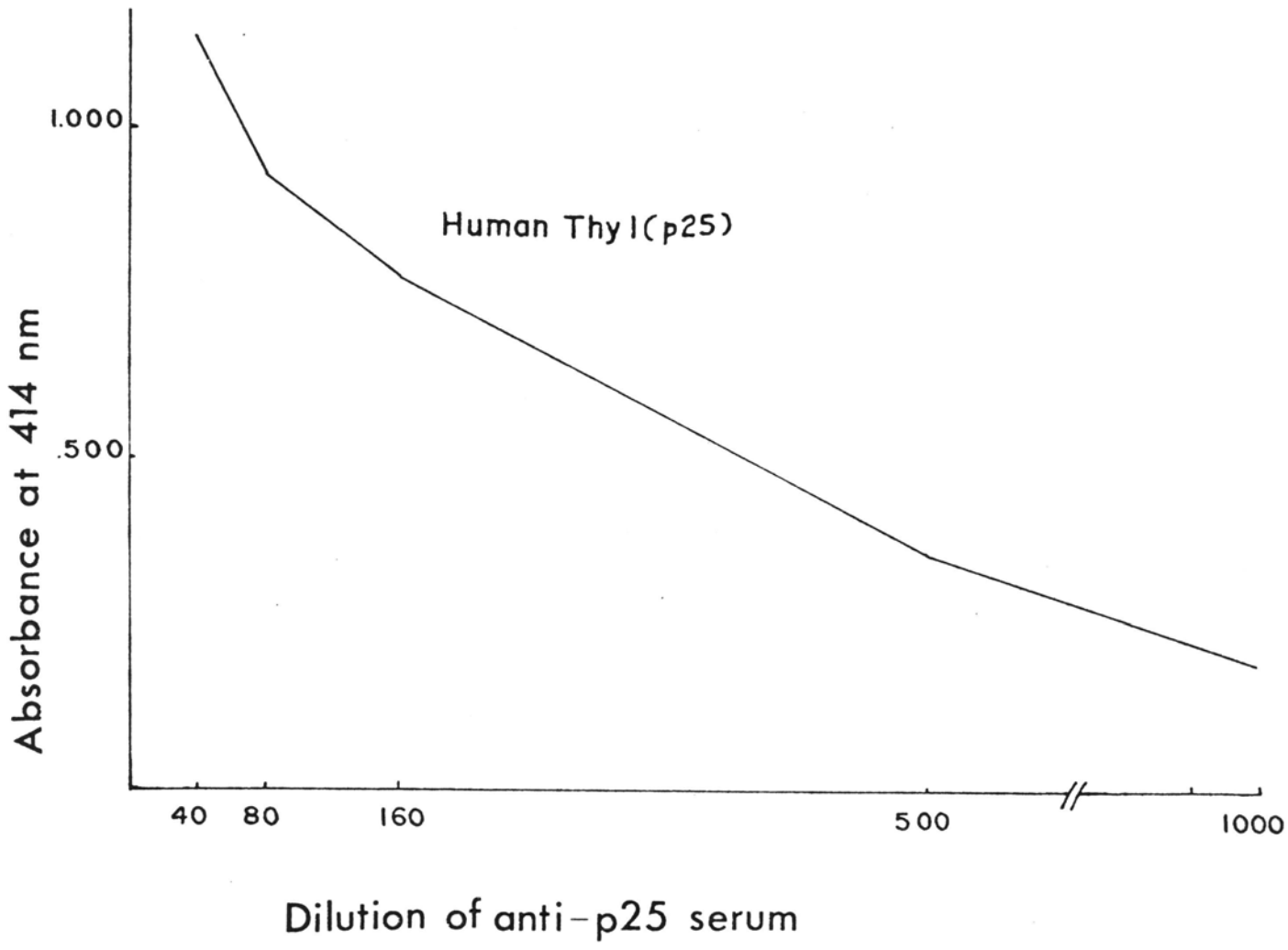
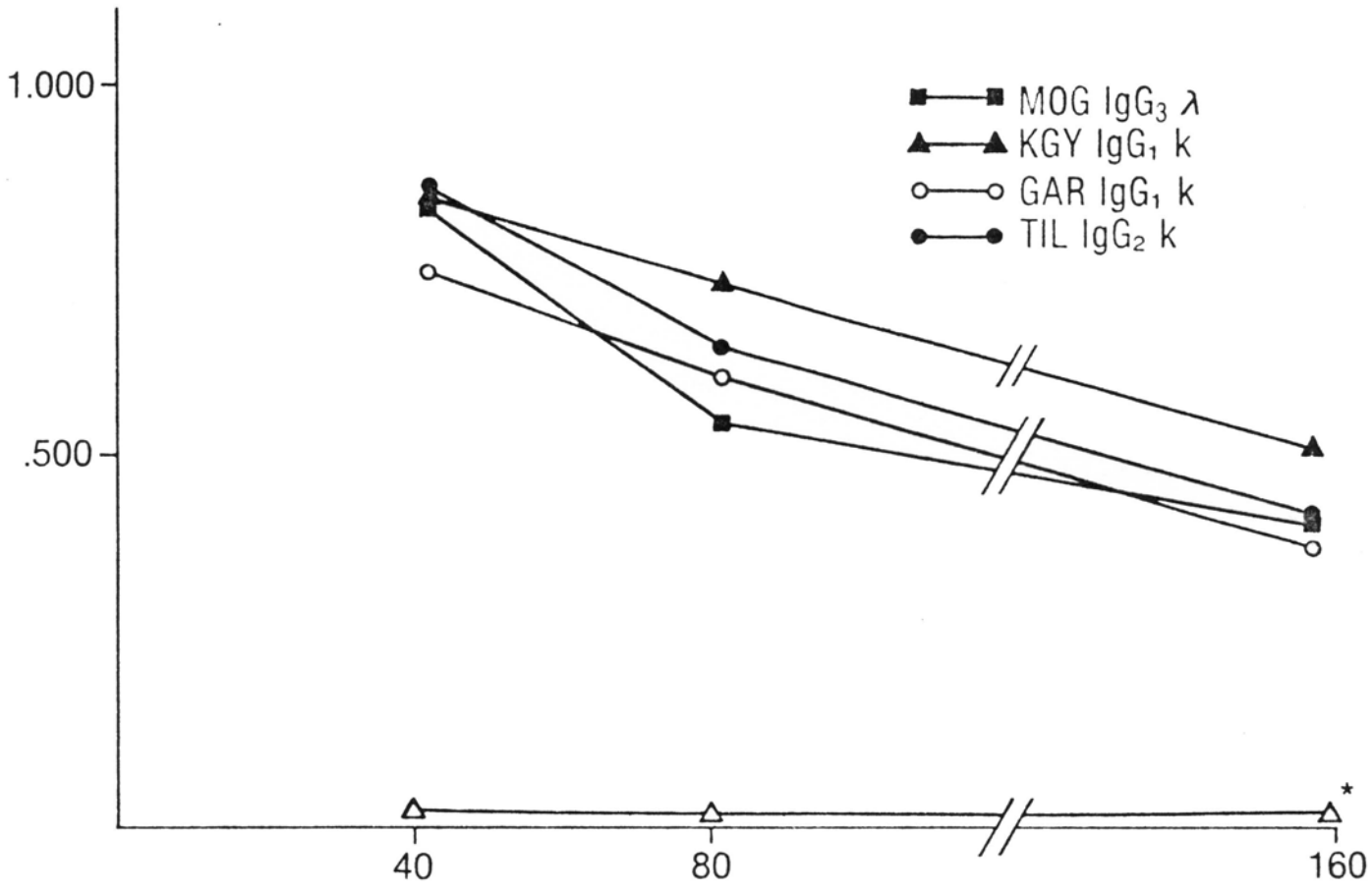

1.000
.500
Absorbance at 414 nm
Human Thy 1 (p25)
40
80
160
500
1000
Dilution of anti-p25 serum

Fig. 2. ELISA assay showing reactivity of four human IgG molecules with anti-p25 antisera.



*Ig of chicken, sheep, cow, rhesus, baboon, gorilla, human CAN IgM, JOR IgA, FOR IgA, ANT IgM, NEV IgM, β_2-microglobulin

Fig. 3. Reactivity of rodent immunoglobulins with anti-p25 antisera.

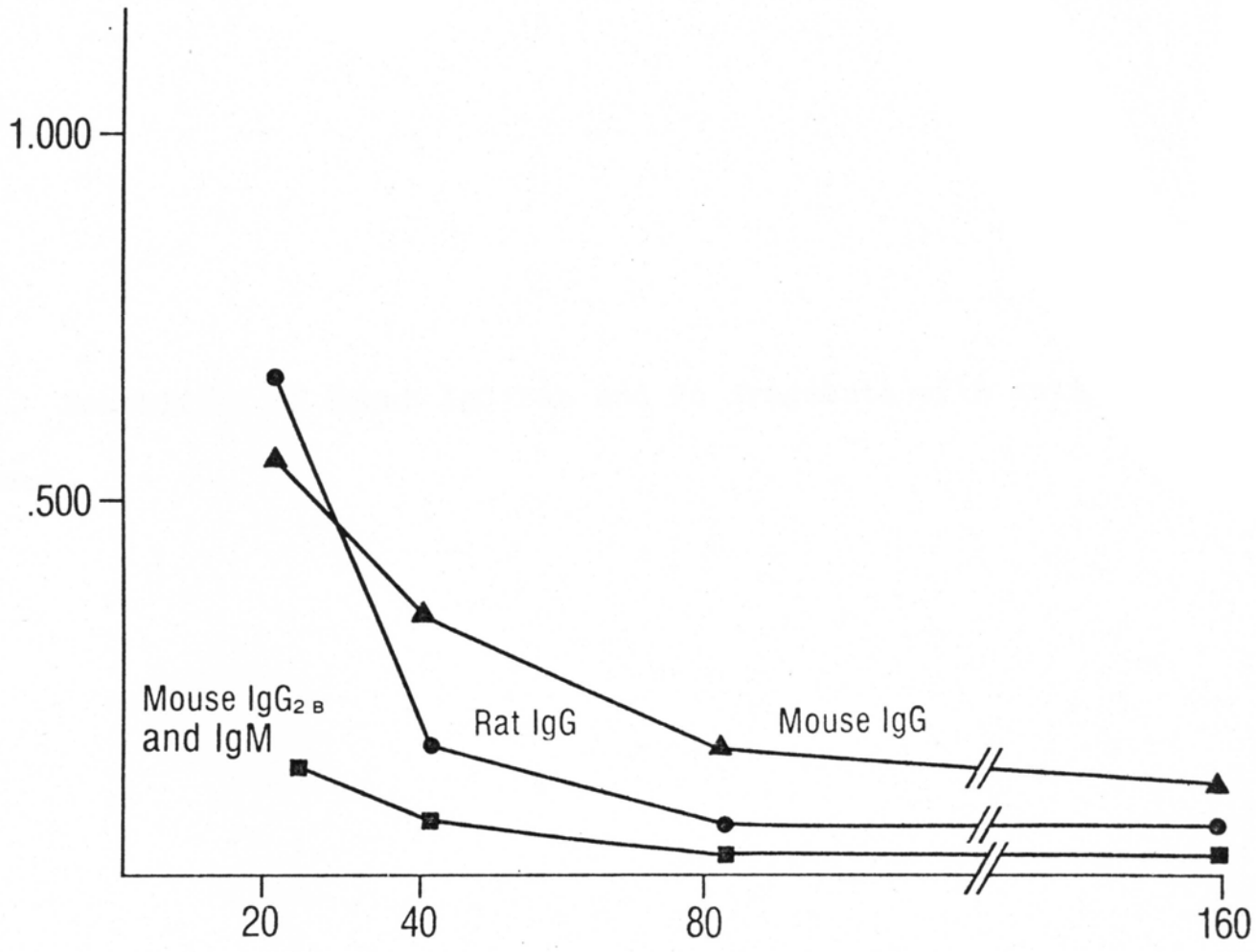

1.000
.500
Mouse IgG2 B
and IgM
Rat IgG
Mouse IgG
20
40
80
160

Fig. 4. Reactivity of human IgG Fab and Fc fragments with anti-p25 antiserum.

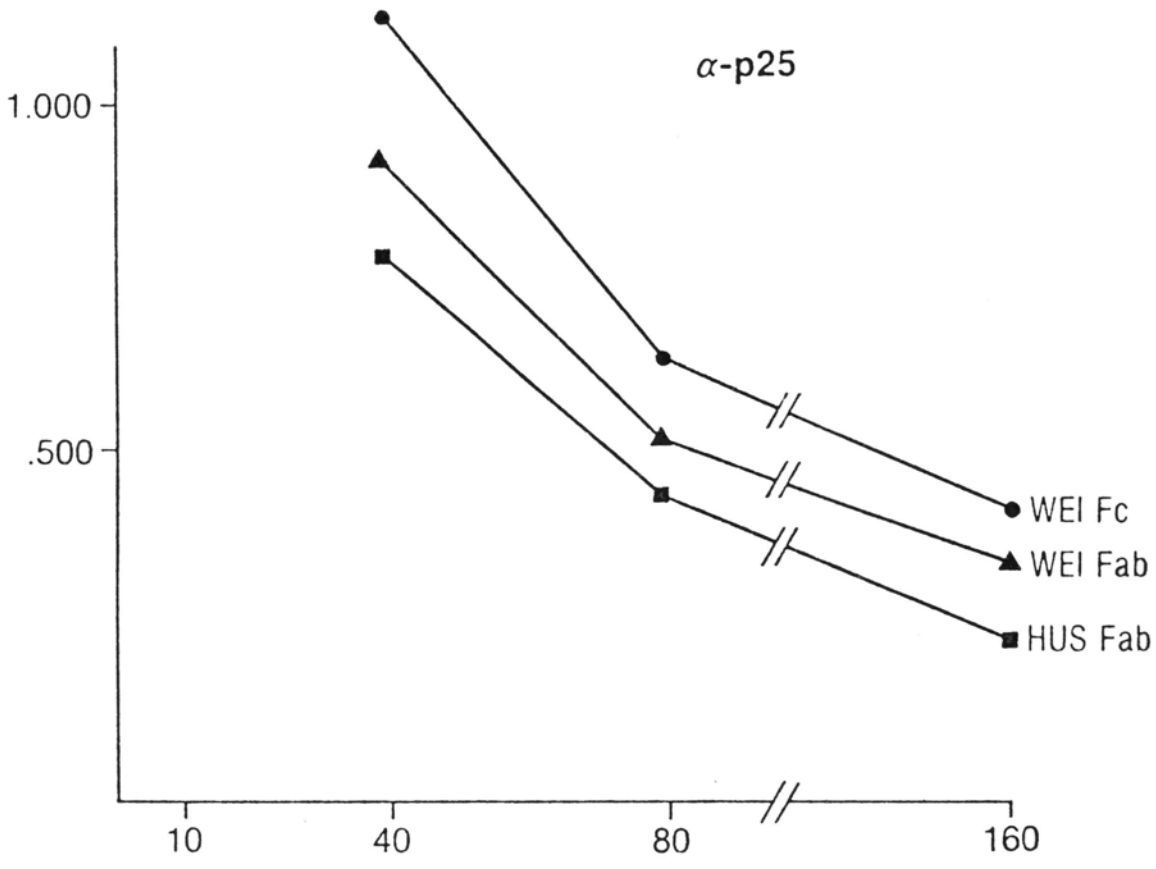

α-p25
1.000
.500
10
40
80
160
WEI Fc
WEI Fab
HUS Fab

Fig. 5. ELISA assay reactivity to human IgG after (A) Reactivity of four human IgGs with unabsorbed anti-p25 antiserum plus reactivity to a control of normal rabbit serum. (B) Reactivity to the IgG's with anti-p25 antiserum absorbed with normal pooled human IgG. (C) Reactivity of human IgG to anti-p25 antisera after absorption with Molt 4 cells ($1x10^6$). (D) Reactivity of human IgG with acid wash of the IgG coupled Sepharose 4B column as seen in B.

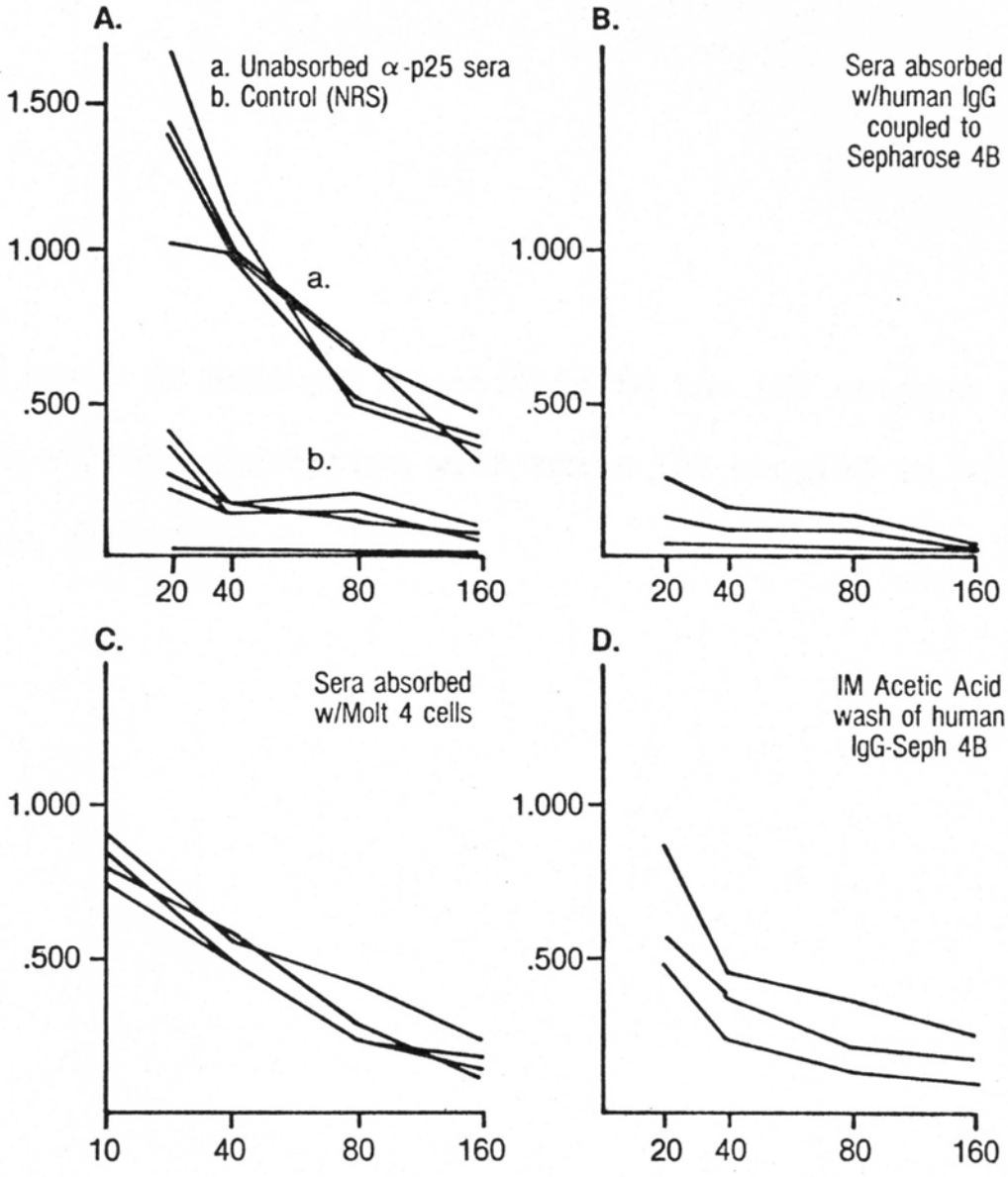

A.
a. Unabsorbed α-p25 sera
b. Control (NRS)
1.500
1.000
.500
a.
b.
20 40 80 160
B.
Sera absorbed w/human IgG coupled to Sepharose 4B
1.000
.500
20 40 80 160
C.
Sera absorbed w/Molt 4 cells
1.000
.500
10 40 80 160
D.
IM Acetic Acid wash of human IgG-Seph 4B
1.000
.500
20 40 80 160

Fig. 6. ELISA assay of anti-p25 reactivity to the p25 antigen after absorption with Molt 4, absorption with human IgG coupled to Sepharose 4B, or 1M acetic acid wash.

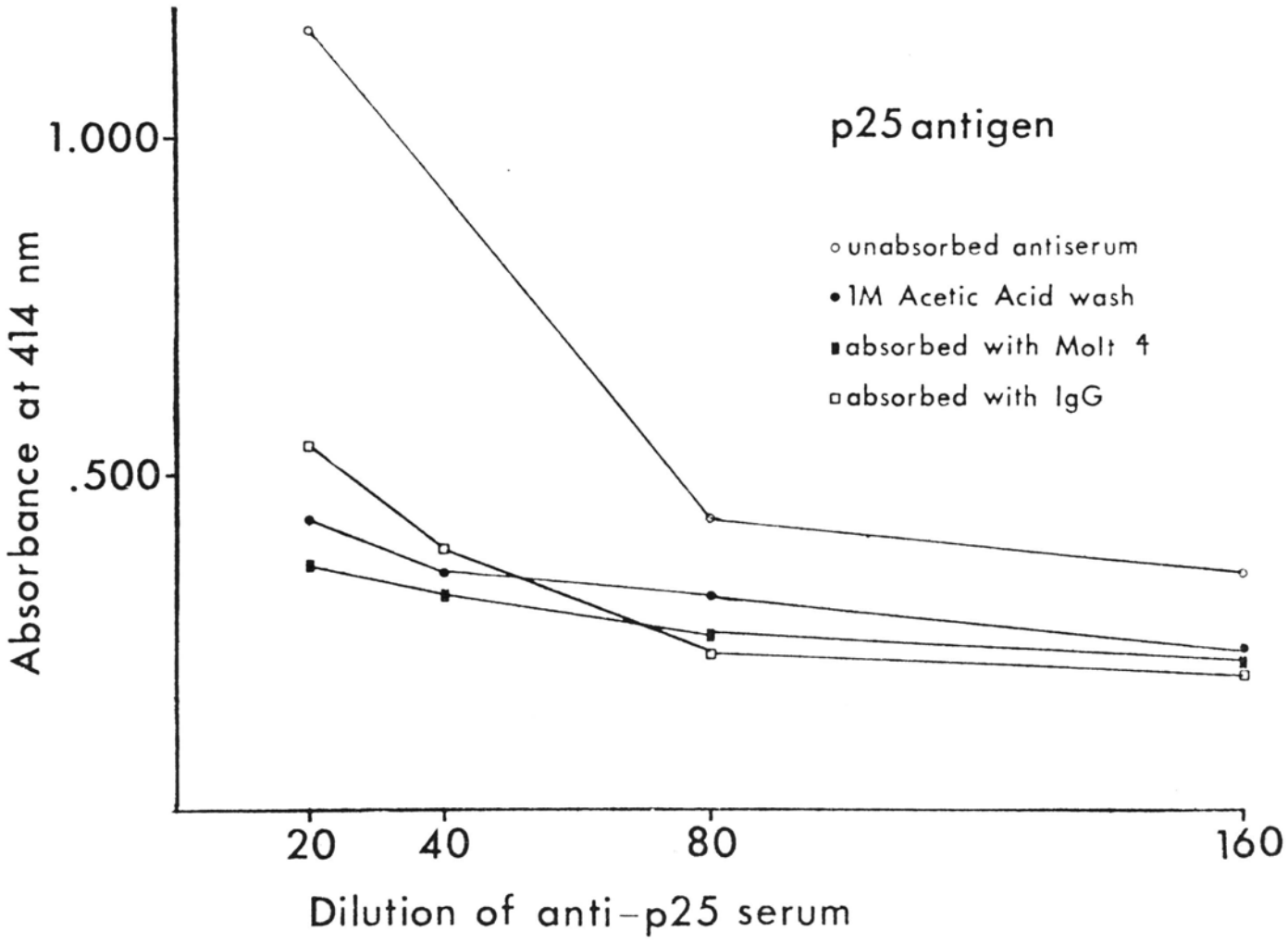
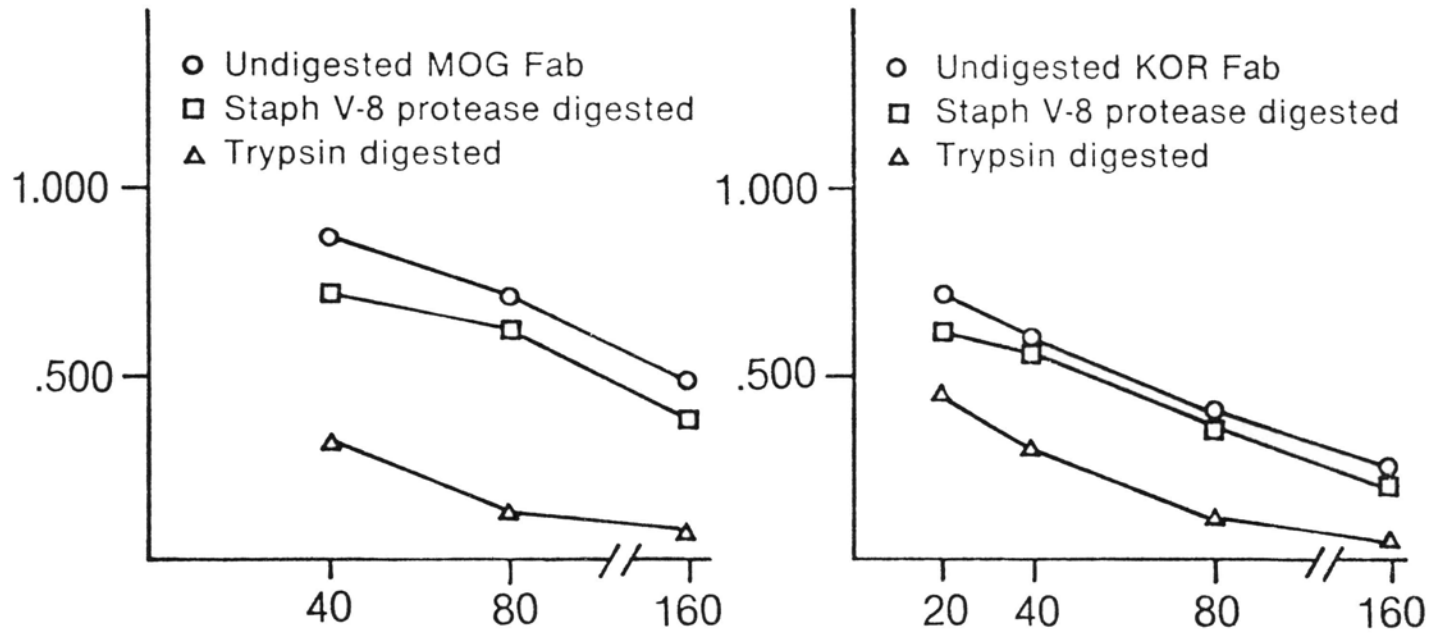

p25 antigen
unabsorbed antiserum
1M Acetic Acid wash
absorbed with Molt 4
absorbed with IgG
1.000
.500
Absorbance at 414 nm
20
40
80
160
Dilution of anti-p25 serum

Fig. 7. Reactivity of trypsin digested, Staph V-8 protease digested, and intact IgG Fab fragment with the anti-p25 antiserum.

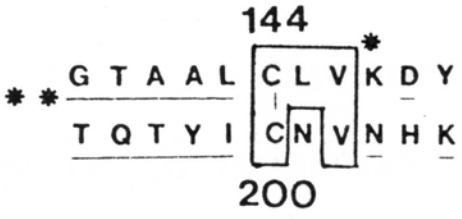


Dilution of anti-p25 serum

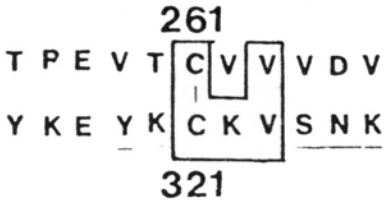
Fig. 8. Amino acid sequence surrounding disulfide bonds for the constant domains of human IgG and the 9-112 disulfide bond of murine Thy-1.

Human IgG

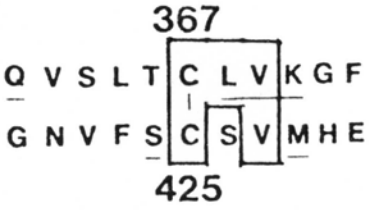
C_H1 Domain



C_H11 Domain



C_H111 Domain

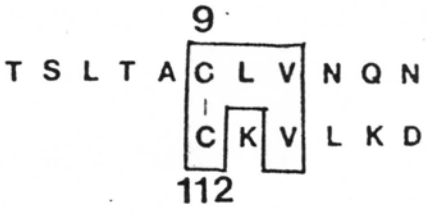


*Enclosed boxes denote similarity to murine Thy I disulfide bond

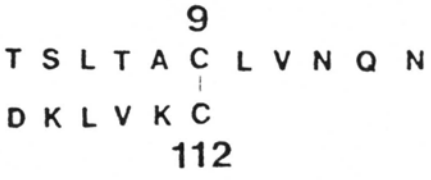
* *Underlined are the amino acids that are different in the rabbit

Murine Thy 1

Proposed Dimensional Structure[1]



Alternate



[1] Fits William and Gagnon's model for murine Thy I

CHAPTER 4

BIOCHEMICAL CHARACTERIZATION OF THE HUMAN THY 1

ABSTRACT

The human Thy 1 homologue was further characterized biochemically by sequence and carbohydrate studies. Two other forms of the human Thy 1 molecule were detected and partially characterized. A 40,000 MW molecule is the dimer of the 25,000 MW Thy 1 molecule and its formation is increased by the presence of sodium dodecyl sulfate. The second form of 16,000 MW appears to be a cryptic or breakdown form of the 25,000 MW human Thy 1 molecule.

INTRODUCTION

The Thy 1 or theta antigen was first detected on murine brain and thymus as a cell surface alloantigen (1). Thy 1 can only be isolated in micelle formation using detergent. Rat brain Thy 1 carbohydrate composition has been determined (2) and the protein portion has been sequenced (3), as has been done for mouse brain Thy 1 (4,5). The rodent Thy 1 has been shown to consist of approximately 111 amino acids, it contains four cysteines and also contains three N-linked sugar chains. An unusual feature of the glycoprotein is the C-terminal peptide, as it does not contain any extended sequence of hydrophobic amino acids necessary for membrane anchorage (3).

Human Thy 1 (also designated p25) has been isolated from a human lymphoblastoid T cell line, Molt 3, and characterized by antigenic studies using cross-reactivity and immunoabsorption experiments with mouse Thy 1.2 (6). Human Thy 1 has the same molecular weight as its murine counterpart according to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (7). The human Thy 1 molecule was found to be similar though not identical to its murine homologue by peptide mapping and amino acid composition. The same types of comparisons have been made and similar conclusions drawn using Thy 1 glycoprotein isolated from human brain and fibroblasts (8).

In our present studies, we have detected two other forms of the human Thy 1 antigen. One form is approximately 40,000 daltons in MW (molecular weight) according to SDS-PAGE and is designated p40 and the other is 16,000 daltons MW, designated p16. Both forms are reactive

with antisera made to the human Thy 1 or p25 antigen. The p40 and p25 seem to have almost identical amino acid compositions. The amino acid composition of the p16 is also very similar. The carbohydrate composition of the p40 and p25 are similar while the p16 seems to have less carbohydrate. HPLC (high-pressure liquid chromatography) chromatograms of tryptic digests of the p40 and p25 in their monomeric and in their aggregated forms are identical. The p16 tryptic peptide chromatogram is different but seems to contain a number of identical peptides. Therefore, the p25 antigen appears to form as a dimer the p40 created during isolation; its formation is accelerated by exposure to SDS; and the Thy 1 may also exist in a breakdown form that is possibly present on the cell surface.

MATERIALS AND METHODS

Isolation: Molt 3 cells were grown according to procedures described previously (9) and isolation of the p25 antigen was performed as described previously (6) with the following modifications. After elution with 3% alpha-methyl mannoside of the Sepharose 4B *Lens culinaris* column (Vector), the bound fraction was applied to a S-200 Superfine column and the low molecular weight (LMW) fraction was applied to a preparative SDS-PAGE and extraction of protein was performed as described previously (7). The purified protein was concentrated by lyophilization and applied to a 12 cm x 0.5 cm Sephadex G-25 column in distilled water. This step was deemed necessary in order to remove contaminating glycine from the Tris-glycine SDS-PAGE and other contaminants which interfered with carbohydrate analysis (See Figure 1).

In one isolation procedure the preparative SDS-PAGE was fixed in a 50% Trichloro-acetic acid (TCA), 0.1% Coomassie Blue solution. The 25,000 and 40,000 MW bands were excised and the protein extracted from the gel as described previously (7).

The 25,000, 40,000 and 16,000 MW material was also isolated from an immunosorbant column prepared essentially according to the method of Marsh *et al*. (10). The 1M glacial acetic acid fraction was applied to a preparative SDS-PAGE and the protein extracted as previously described (7).

Analytical SDS-PAGE was performed according to the method of Laemmli (11), using a 10% or 12.5% gel on a Hoefer electrophoresis apparatus. The gels were stained with Coomassie Blue. If the bands were not visible by this stain, the gel was subsequently stained by the silver-nitrate method (12).

Complete reduction of proteins was performed by addition of 10% β-mercaptoethanol and heating at 100°C for 5 min. in a heating block before application to SDS-PAGE.

Production of Antisera: The antisera was raised as described previously (6). Briefly, rabbits were injected intramuscularly with the p25 antigen in complete Freund's adjuvant. Second and third injections followed 14 and 28 days later with incomplete Freund's adjuvant.

Enzyme-linked Immunoadsorbant Assay, (ELISA): This assay was performed as described previously (13).

Amino Acid Composition: The amino acid (a.a.) compositions of purified p40, p25, and p16 antigens were analyzed by standard procedures using a Durrum D-500 automatic amino acid analyzer. Approximately 50 ug of protein was hydrolyzed in 6N HCl, 0.1% phenol at 110°C under vacuum for 18 hrs. Sperm whale myoglobin was used as a control and to correct a.a. composition calculations as described previously (7).

Peptide Graphs of Tryptic Digests on HPLC: Tryptic digestions were performed as described previously (7). Separation of peptides was accomplished on a Waters High-Pressure Liquid Chromatograph, using an

ODS-Hypersil reverse phase column (Altex) essentially according to the parameters of Yang et al. (14). A linear gradient was run using a pH 2.14 triflouro-acetic acid in distilled water as Buffer A and a 40% buffer A and 60% acetonitrile as Buffer B. The gradient was increased from 0-70% over 50 minutes at a flow rate of 1.5 ml/min while maintaining the column temperature at either 45°C or 60°C. The eluate was read at 220 nm using a Kratos variable wavelength spectrophotometer.

Carbohydrate Composition: Identification and quantitation of neutral and amino sugars was determined by the alditol acetate method (15,16). Percent carbohydrate calculations were made according to the formula of Sloneker (17). Gas chromatography was performed on a Hewlett-Packard Model 5830A gas chromatograph equipped with dual flame ionization detectors. A 6 ft x 2 mm glass column packed with 3% SP-2340 was used. Injection temperature was 250°C and detector temperature was 350°C. Initial temperature was 190°C, held for 6 min then raised to a final temperature of 240°C at a rate of 1.7°/min. and held for 20 min. The carrier gas was nitrogen at 20 ml/min. Peak areas were measured and integrated by a Hewlett-Packard 18850A GC terminal.

Approximately 100 ug of the samples p40, p25, and p16 were hydrolyzed in 0.5 mls of 3N HCl at 80°C for 16 hrs. The samples were extracted 3X with 0.5 ml of n-hexane. Inositol acetate was added as an internal standard. The sample was incubated for 1 hr at room temperature after the addition of 0.5 ml of 1N NH_4OH containing 2 mg $NaBH_4$. The boric acid was then neutralized with acetic acid (app. 7 drops).

One ml of methanol:benzene (5:1) + 50 ul acetic acid was added to the sample and it was incubated at 80°C for 5 min. and then evaporated under nitrogen. This step was repeated 4X without benzene. The sample was completely dried and acetylated with 0.75 ml acetic anhydride at 100°C, under nitrogen for 1 hr, after which 0.5 ml of chloroform was added to the sample and extracted 5X with 0.5 ml distilled water. The sample was dried and reconsituted in an appropriate volume of chloroform for injection.

Cyanogen Bromide Cleavage: Approximately 20 ug of the p25 antigen was dissolved in 20 ul of 70% formic acid, after which 20ul of a cyanogen bromide solution (25 mg Cnbr/1 ml 70% formic acid) was added. The sample was incubated at room temperature for six hours at which time 1 ml of distilled water was added and the sample was lyophylized.

Amino Acid Sequence Determination: N-terminal amino acid sequences were determined by Edman degradation with a Beckman Model 890 C automatic protein sequencer using a 0.1 M quadrol buffer. Identification of amino acid phenylthiohydantoin derivatives was carried out with a Dupont high pressure liquid chromatograph.

Immunoblot Technique: Immunoblotting was performed essentially according to the method of Tsang et al. (18) with modifications in the staining procedure. Briefly, a DOC and a SDS solubilized membrane preparation of Molt 3 cells were electrophoresed on a 1.5 mm thick slab gel. The gel was then transblotted against a nitrocellulose transfer blot (Bio-Rad) overnight at 300 ma at 4°C in blotting medium, 0.025 M Tris, 0.193 M glycine, in 20% methanol. The nitrocellulose was washed

3X with 1% gelatin in PBS for 15 min. and then the anti-p25 antibody (1:100 dilution in 1% gelatin) was applied for 2 hrs under gentle shaking. The blot was then washed once in 1% gelatin/PBS and the secondary antibody [Goat anti-rabbit horse-radish peroxidase coupled (Cappel) 1:1000 in 1% gelatin] was applied for 2 hrs. The blot was then washed in PBS 2X for 15 min before applying the substrate, 0.05% 4 chloro-1-napthol in PBS plus 0.1% H_2O_2 (3%) for 20-30 min. The blot was then washed with H_2O and air dried.

RESULTS

The major band seen after isolation of the LMW peak from the S-200 Sephadex column was the p25 (see Fig. 2A). Concentration of the p40 and the p16 vary from preparation to preparation from undetectable to 10-20% of total protein. Desalting on the small Sephadex G-25 was found necessary in order to remove glycine and other contaminants which interfered with amino acid and carbohydrate compositions.

As can be seen in Table 1, the a.a. composition of the p40 and p25 are almost identical, as is the p16, though slightly different. These compositions are very similar to those reported for mouse Thy 1 (4), rat Thy 1 (2) and human brain Thy 1 (8). By using the method to determine relatedness among proteins of Marchalonis and Weltman (19) which is based upon statistical analysis of differences in amino acid composition, we determined that the p25 and p40 are most closely related to the murine Thy 1 (see Table 2). The p25 a.a. composition was compared to that of 168 other proteins of which those proteins shown in Table 2 are representative.

Figure 3 shows the relative migration distance of the p40 and the p25 on a 12.5% SDS-PAGE stained with Coomassie blue.

The ELISA assay showed that all three antigens are reactive to antisera made against the p25. The p40 is more reactive than the p25 which is in turn more reactive than the p16 to antisera made to the p25 antigen as shown in Figure 4.

When the p25 and the p40 were run on a preparative SDS-PAGE and then fixed in the TCA-Coomassie blue fixing solution, then extracted, and rerun on a 10% analytical SDS-PAGE, both molecules migrated identically. As can be seen in Figure 5, the now aggregated p25 and p40 migrated at an approximately 110,000 MW range.

When the aggregated p25 and aggregated p40 were treated with a reducing agent, ß-mercaptoethanol, both reduced to the p40 form of identical migration on SDS-PAGE (Figure 6). When the p40 molecule is reduced it is not converted to the p25 form (data not shown). The relative migration of the p16 molecule is shown in Figure 6d. Some microheterogeneity is seen as is characteristic of glycoproteins.

When the p40, p25, and p16 were digested with trypsin and run on high pressure liquid chromatography at 60°C, the chromatograms appeared very similar for the p40 and the p25, yet, slightly different for the p16 (Figure 7). Twelve to fourteen peptides are seen as would be expected in a tryptic digest of the Thy 1 antigen.

HPLC chromatograms of tryptic digests of the aggregated p40 and aggregated p25 also revealed the identical nature of these molecules (Fig. 8). The tryptic peptide maps of the aggregated molecules appear different from those of the non-aggregated molecules. This is because the aggregated maps were run under a temperature of 45°C while the non-aggregated materials were run at 60°C. The increased temperature induced the peptides to be eluted sooner. The approximate number of 12-14 peaks remains the same for all proteins.

Amino acid sequence determination of approximately 2 nmole of reduced and alkylated p25 revealed no PTH amino acid. It is likely the n-terminal amino acid of the molecule is blocked. Sequence determination of a cyanogen bromide peptide of p25 revealed a serine at the amino terminus.

Carbohydrate analysis was performed using 100 ug of the p40, p25, and p16 antigens. The antigens were desalted on the G-25 Sephadex column before running on gas chromatography. Table 3 shows that the percentages of the mannose, galactose, glucose, and glucosamine are similar for the p40 and p25 antigens. However, the percentages for the p16 were quite different with no glucosamine detected.

The presence of SDS seems to convert the p25 to the p40 molecule. In order to prove p25 conversion to the p40 form was induced by SDS, a membrane preparation of Molt 3 cells was dissolved in either 2.5% DOC or 2.0% SDS 10 mM Tris-HCl. The immunoblot showed p25 and p40 in the DOC preparation and the absence of p25 with greater amount of p40 in the SDS preparation along with actin and some high molecular weight material, perhaps the 110,000 MW aggregate (Figure 9).

Molt 3 membrane preparations solubilized in DOC showed the presence of p25 by immunoblot, however the same preparation solublized in SDS did not show any detectable p25 (Figure 9). Both contained p40, actin, and assumed aggregated material. The anti-p25 serum has been shown to be slightly reactive to actin (manuscript submitted). In a membrane preparation, actin is present in a much greater amount than the p25 molecule, therefore appearing as a much darker band on the immunoblot.

DISCUSSION

The Thy 1 molecule is a glycoprotein found on rodent thymocytes, T lymphocytes, neuronal cells, and fibroblasts. It has long been known as a marker for T cells in mice. The molecule has been sequenced in the mouse and rat brain and characterized according to the carbohydrate moieties (2-5). Homologues have been found in man (6,20), dog (21), and chicken (22). However, the antigen still remains a puzzle in several respects. For example, although predominantly found on neuronal cells, the expression of Thy 1 on lymphoid cells varies according to species. Thy 1 is abundant on mouse and rat thymocytes, and present on mouse T lymphocytes, yet absent from rat T lymphocytes (2). At present, there is a great deal of confusion and disagreement in the literature as to where Thy 1 can be found in the human, and whether it is present on human thymocytes or T lymphocytes at all. According to McKenzie and Fabre (23) it is only present in discrete areas of thymus, spleen, and lymph nodes, but according to Balch and Ades (24), it is expressed on thymus and peripheral T lymphocytes. This discrepancy is probably due to the specificity and method of production of antisera in each laboratory. These differences can only be resolved by isolation of the antigen from the tissues in question, followed by biochemical characterization of the isolated antigen.

We have shown in previous work (6) that the p25 antigen isolated from Molt 3, a human lymphoblastoid T cell line, is antigenically cross-reactive with mouse Thy 1, has a similar molecular weight, binds to *Lens culinaris* lectin, has a similar amino acid composition, and is

similar by peptide mapping, though not identical (7). Present studies indicate that the p25 has 12-14 tryptic peptides which is similar to the number seen with tryptic digests of murine Thy 1: the p25 is approximately 20% carbohydrate which is similar to rodent Thy 1 (3,5,20); and sequence studies suggest that the amino terminus is blocked by a pyro-glutamate as is present in the rodent Thy 1 (3,5). Amino acid sequencing of a peptide created by treatment of the p25 molecule cyanogen bromide shows the amino terminus to be a serine which is the same amino acid at position 97 following the only methionine at position 96 in mouse Thy 1 (5). This is not seen in the rat Thy 1 which possesses a methionine at position 95 and a cysteine at position 86 (3).

During isolation of the p25 antigen we noticed bands of lesser amount in the 40,000 MW range that usually could not be completely removed from a p25 preparation, yet a 16,000 MW that could. These bands eluted from preparative SDS-PAGE were found to react with antisera made to the p25 antigen and were also shown to have very similar amino acid composition and tryptic digest peptide maps from HPLC.

It is likely that the p40 is the p25 dimerized. When the p25 and p40 were eluted from preparative SDS-PAGE that had been stained and fixed in a TCA-Coomassie blue solution, the molecules aggregated to approximately 110,000 daltons and appeared identical, therefore also indicating that the p25 and p40 are the same molecule. We assume that the p40 is the result of dimerization of the p25 as a result of

exposure to SDS and is therefore a product of isolation. The dimerization of the p25 to the p40 form could not be due to disulfide-bond interlinkage because it is not reduced by mercapthoethanol. Perhaps a hydrophobic moiety on the molecule is causing the dimerization. We do not know whether the p40 can be found on the cell membrane.

Ours is not the first study to detect molecules of differing molecular weight which are reactive to anti-Thy 1 antisera. Saji and Tanigaki (25) isolated a Thy 1 like antigen from a human T-cell leukemia cell line SKW-3. They detected a major component of 30,000 MW and another of 110,000 MW which reacted with their rabbit antiserum made against a Molt 3 glycoprotein. Our reduced and alkylated p25 antigen also migrates at approximately 29,000 MW on SDS-PAGE. Perhaps this 110,000 MW molecule is related to the aggregated p25 and aggregated p40. Kuckel reported (26) a 230,000 MW for rat Thy 1 in the absence of deoxycholate.

Sauser and co-investigators in 1974 (27) isolated a 40,000 MW protein from Swiss albino murine thymocytes. When this protein was injected into AKR mice, anti-theta alloantiserum was produced. This 40,000 MW protein was isolated in the following manner. A membrane fraction was stabilized in a Tris-HCl, 2% SDS buffer and centrifuged. The supernatant was applied to a Sephadex G-200 column and the fractions were dialyzed against distilled water. This differs from the accepted protocol of isolation of murine Thy 1 which includes dissolving the membrane fraction in a 2.5% DOC Tris-HCl buffer, centrifugation, and the supernatant applied to a lectin column (usually

lens culinaris) and then gel filtration. We propose that utilizing different solubilization techniques and a different detergent, SDS instead of DOC, causes the murine Thy 1 to dimerize to a 40,000 MW. Support for this proposal comes from immunoblots of Molt 3 membrane preparations using either SDS or DOC. In contrast to the DOC preparation, no p25 was detected in the SDS preparation.

Since Thy 1 is found on neuronal cells at synaptic junctions of the brain and since T lymphocytes are regulators, i.e., helpers and suppressors of the immune system, Thy 1 could be functioning in cell-cell communication. Seeds and his coworkers (28) have correlated the presence and absence of Thy 1 with the presence and absence of synapses in cultures of brain aggregates. Williams and his group (29) have shown that anti-Thy 1 sera applied to brain can affect rat behavioral responses. These antibodies also inhibit virus-dependent proliferation of leukemia cells (30). By comparing sequence homologies of rat and mouse Thy 1 with immunoglobulin domains, HLA antigens, and $b_2$microglobulin, Williams and Gagnon (5) have proposed that the Thy 1 is the best candidate for a molecule which may approximate the primordial immunoglobulin domain. Thy 1 could function as a receptor for another molecule or one could argue that Thy 1 is a ligand to be recognized by receptors on other cells.

Since the p16 antigen reacted to antisera and possessed similar amino acid composition to the p25 antigen, it is possible that the different migration on SDS-PAGE could be due to reduced amount of carbohydrate. The molecular weight of rodent Thy 1 has been determined

to be 17-18,000 daltons by sedimentation equilibrium studies (26) and to be approximately 20-30% carbohydrate (2,4). It is because of this increased carbohydrate content, which affects the mass/charge ratio of the molecule, that the Thy 1 appears as 25,000 MW on SDS-PAGE. If carbohydrate was reduced, the p25 molecule could theoretically migrate in the area of the p16 molecule.

Carbohydrate analysis did not show the p25 and p40 to be identical, yet this is within the range of error for this type of analysis. The p16 antigen possessed considerably fewer sugar residues compared to the p25. This p16 antigen is assumed to be the p16 antigen found on suppressor cells by Balch and coworkers (24,31). They prepared an anti-monkey thymocyte serum which recognized a 25,000 and a 16,000 MW antigen. The antiserum was absorbed with Molt 4, a T lymphoblastoid cell line which possesses the p25 but not the p16 antigen, thereby making the antisera specific for the p16 antigen. Using the pokeweed mitogen immunoglobulin-induction assay, they showed that a detergent soluble 16,000 MW antigen was expressed on human suppressor T lymphocytes and minimally on helper T lymphocytes. Agthoven (32) found a 25,000 MW protein associated with the T-3 antigen, a 19,000 MW protein, after tritiated labelling of human peripheral blood T lymphocytes and immunoprecipitation. Borst and coworkers (33) found four distinct glycoproteins associated with the T-3 antigen immunoprecipitates from a human leukemia cell line HPB-ALL. One of the complex had a molecular weight of 25-28,000 MW by SDS-PAGE and after deglycosylation with Endoglycosidase F had an apparent molecular weight of 16,000. Therefore this molecule has a 16-k dalton protein backbone and carries

only N-linked, complex-type carbohydrate.

The p16 could be a cryptic antigen, i.e., part of the p25's structure removed by some form of enzymatic degradation. Could it, the Thy 1, be processed in the cytoplasm and inserted in the membrane without the attachment of carbohydrate? Does the p16 antigen play a role in suppressor T cell function or is it merely co-expressed and present as a breakdown product on older T lymphocytes? These questions remain to be answered, and may or may not aid in determining the function of the Thy 1 molecule.

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Table 1

Amino Acid Compositions

Approximate amino acid composition of p40 and p16 as compared to that of p25. Compositions are expressed in mol%; tryptophan and cysteine are not included. Values marked with a star are corrected values. Actual values were higher due to inadequate desalting procedure.

Amino Acid	p25	p40	p16
ASX	12.0	11.6	11.3
THR	6.3	6.7	6.4
SER	6.2	5.5	6.6
GLX	13.4	13.7	11.9
PRO	5.9	6.6	3.8
GLY	3.1	3.0*	3.8*
ALA	5.7	5.0*	4.8*
VAL	8.0	6.3	7.0
MET	1.4	1.3	1.5
ILE	4.2	5.5	5.6
LEU	9.6	9.6	9.9
TYR	2.6	3.0	2.5
PHE	3.6	3.6	4.1
HIS	1.8	2.0	2.6
LYS	7.9	7.9	8.1
ARG	4.8	4.5	5.6

Table 2

Comparison of Human THY 1 to Various Proteins Utilizing SΔQ Calculations

Protein	SΔQ	Protein	SΔQ
Mouse Thy 1.2	34	Kappa variable region	173
Rat Thy 1.1	49	Lambda variable region	228
		Heavy variable region	148
p40 antigen	7	Kappa constant region	151
p16 antigen	13	Lambda constant region	181
Human hemoglobin	184	Human CH1 OU	162
Human β2microglobulin	55	Human CH2	142
Human HLA B-27	135	Human CH3	210
Human C-reactive protein	78	Human CH4	134
Nerve Growth Factor	198		
Actin	90	Human CAU μ	110
Human Serum Albumin	72	Human gamma	114
Cytochrome C	197		
Lysozyme	224	Human J chain	147
Ribonuclease	268	Human complement component C4	65

Calculations were performed according to the method of Marchalonis and Weltman (19). This method determine the relatedness among proteins based upon statistical analysis of differences in a.a. composition. Results have been shown to correlate closely with comparisons based on the sequence of related proteins.

$$S\Delta Q = \sum_{j} (X_{i,j,} - X_{k,j,})^2$$

Where i and k identify the particular proteins which are being compared. Xj is the content of a given amino acid of type j.

Table 3

% Carbohydrate Composition

	p25	p40	p16
fucose	0	0	0
mannose	5.4% (8.3)	4.8% (7.3)	4.3% (6.6)
galactose	1.0 (1.8)	2.2 (4.0)	.2 (.3)
glucose	1.8	3.3	1.2
glucosamine	.5 (1.4)	.6 (1.8)	0
galactosamine	0	0	0
sialic acid	N.D.	N.D.	N.D.

Numbers in parenthesis are carbohydrate residues using published carbohydrate composition for α_1 anti-trypsin as a correction factor (33).

Figure 1. Flow chart of isolation of Human Thy 1.

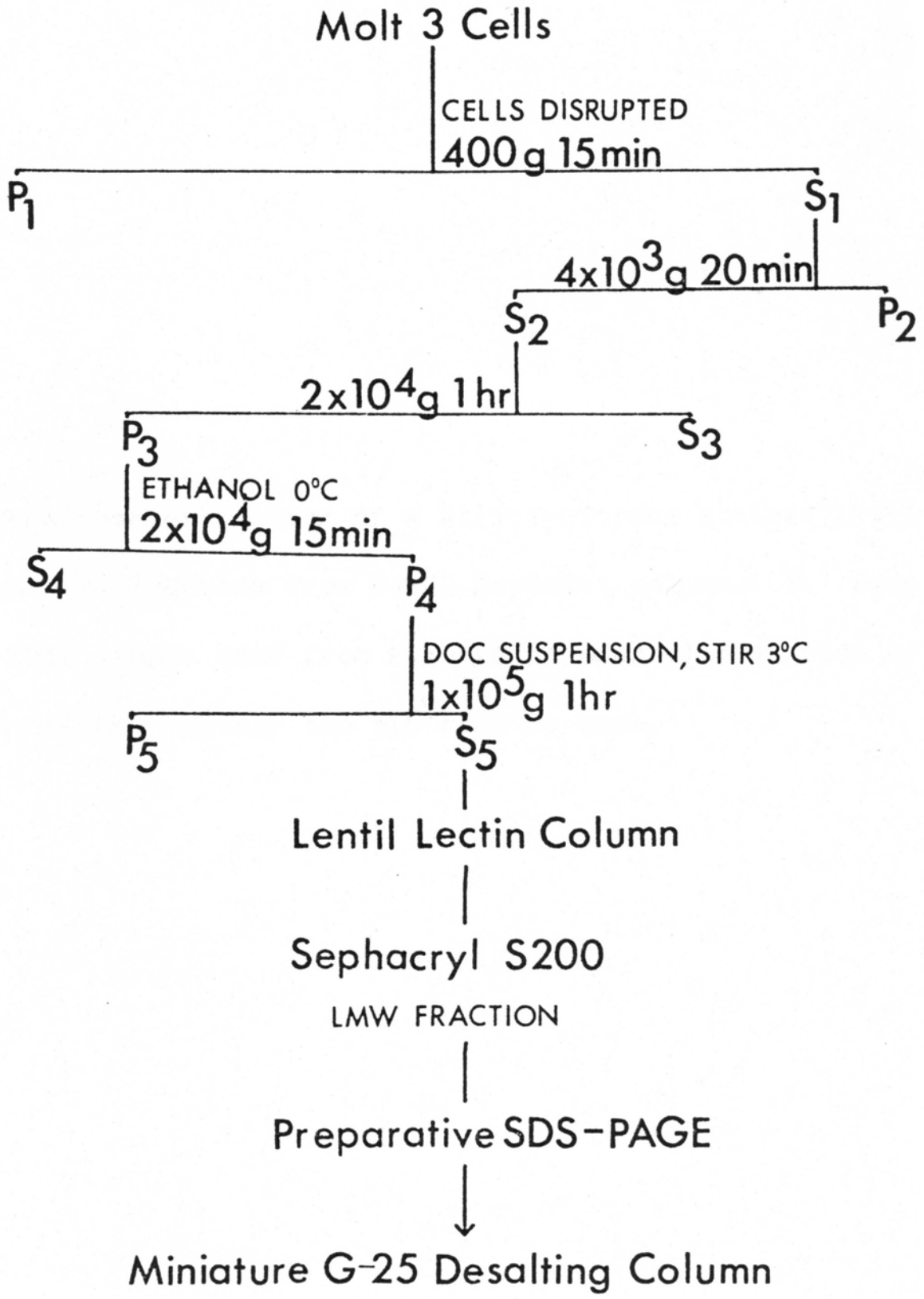
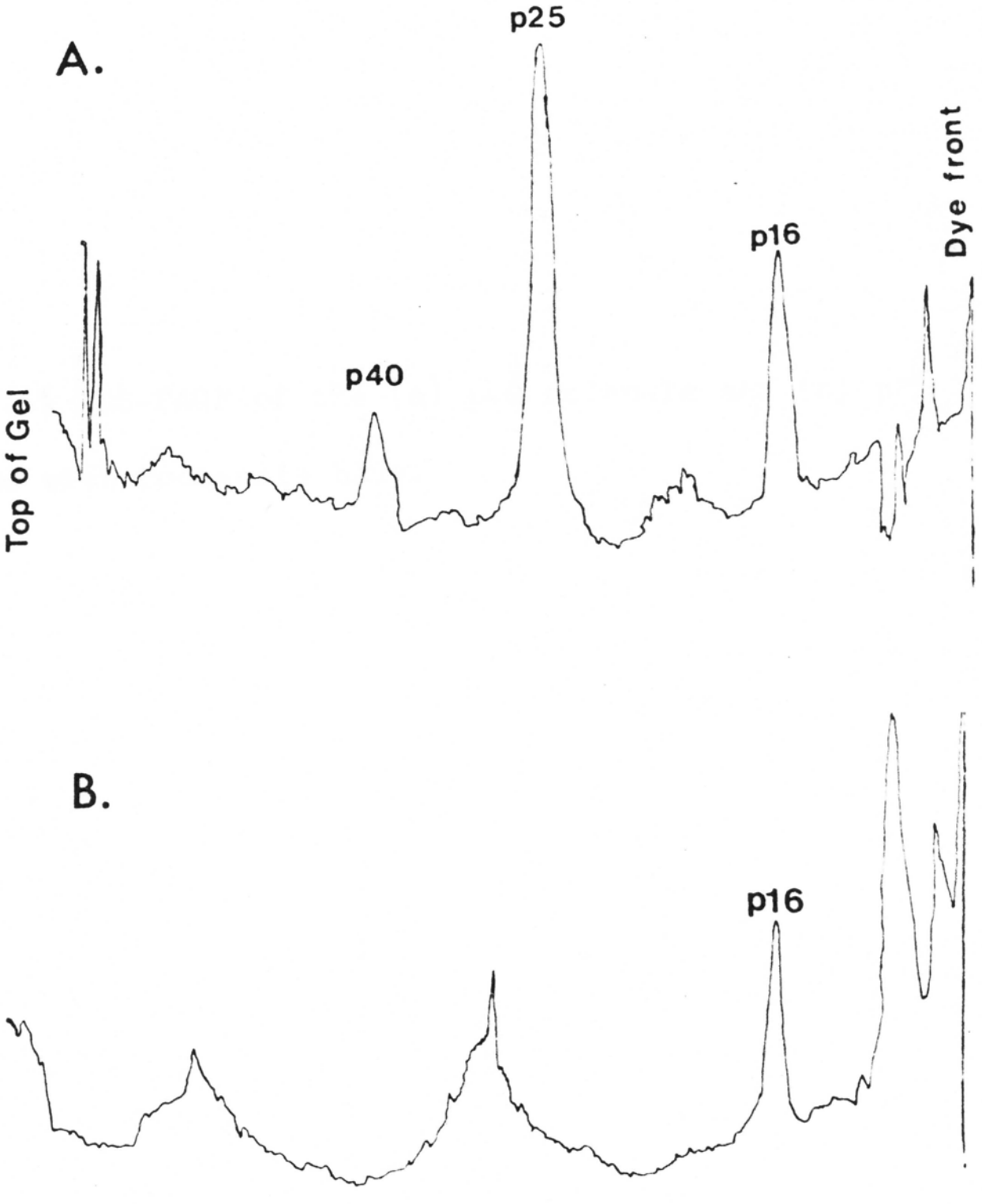

Molt 3 Cells
CELLS DISRUPTED
400g 15min
P1
S1
4x10^3g 20min
S2
P2
2x10^4g 1hr
P3
S3
ETHANOL 0°C
2x10^4g 15min
S4
P4
DOC SUSPENSION, STIR 3°C
1x10^5g 1hr
P5
S5
Lentil Lectin Column
Sephacryl S200
LMW FRACTION
Preparative SDS-PAGE
Miniature G-25 Desalting Column

Figure 2A. Densitometer tracing of a silver-nitrate stained 12.5% SDS-PAGE of the LMW fraction from S-200 Sephadex column. B. Same as above except this sample came from a recycled mannoside elution of the lentil lectin column. Mainly the p16 band is seen.



LMW fraction from G-200 column

Figure 3. A 12.5% SDS-PAGE of the (a) p40 molecule and (b) p25 molecule stained with Coomassie blue.

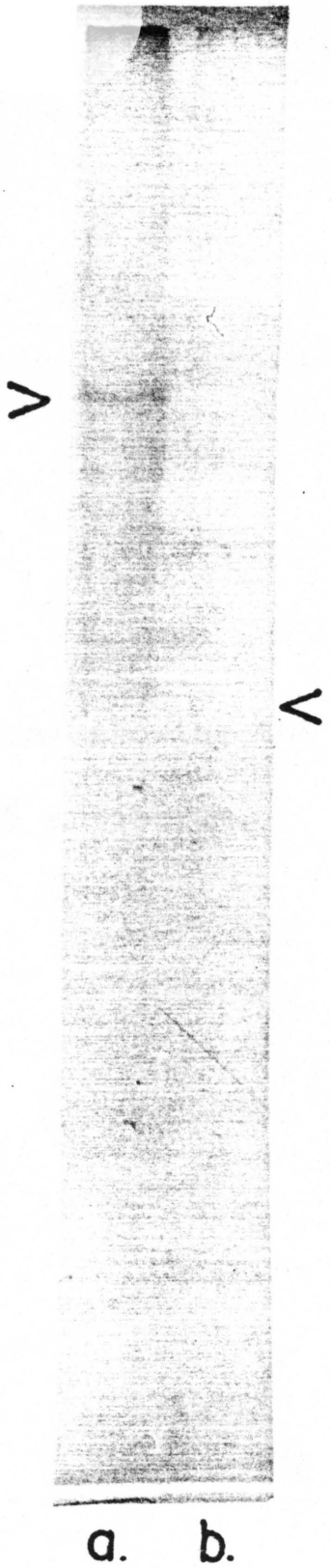

a.
b.

Figure 4. Results of Eliza assay showing the reactivity of the p40, p25, and p16 to rabbit anti-p25 antiserum.

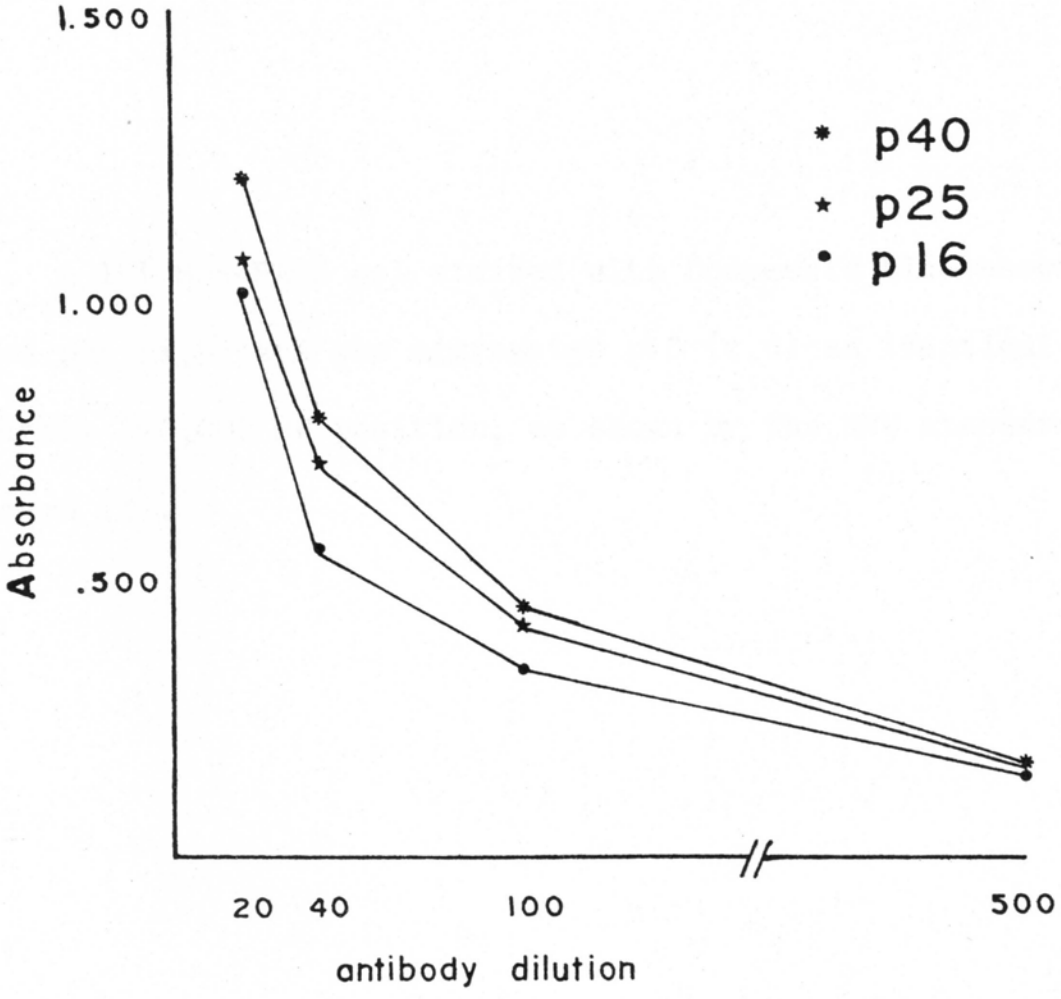

1.500
1.000
.500
Absorbance
* p40
★ p25
• p16
20
40
100
500
antibody dilution

Figure 5. A 10% SDS-PAGE gel stained with Coomassie blue showing the aggregated p25 (a,b) and the aggregated p40 (c,d) as identical bands at approximately 110,000 MW position, as shown by the HMW standard (e) and LMW standard (f).

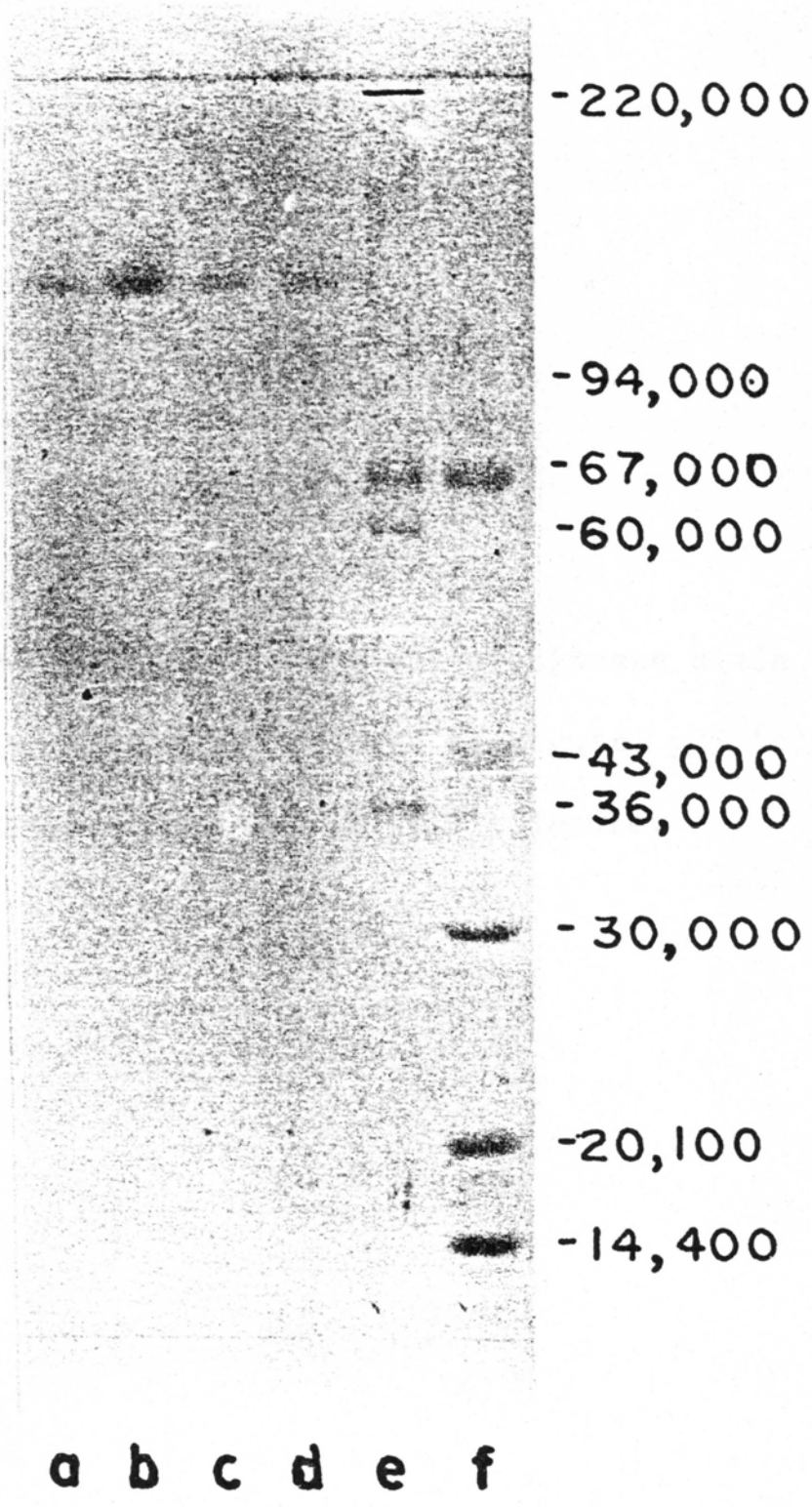

-220,000
-94,000
-67,000
-60,000
-43,000
-36,000
-30,000
-20,100
-14,400
a b c d e f

Figure 6. A 12.5% SDS-PAGE stained with silver-nitrate stain showing the (a) LMW standard (b) completely reduced aggregated p25 (c) completely reduced aggregated p40 and (d) p16 molecule.

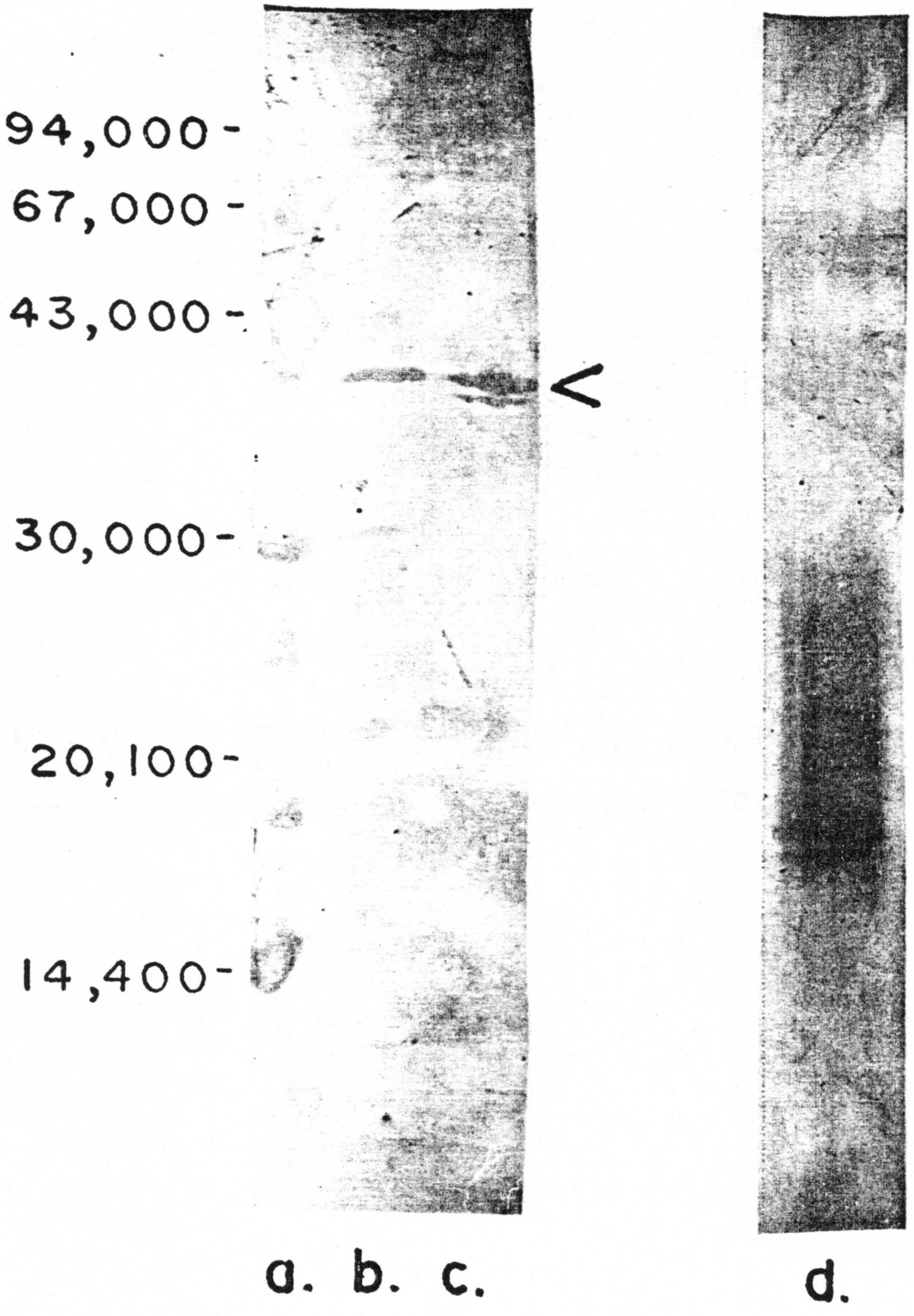
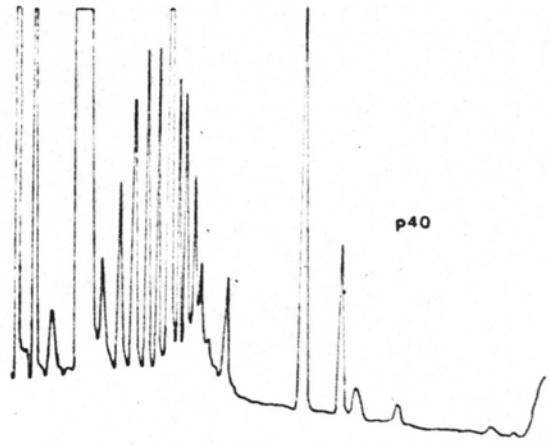
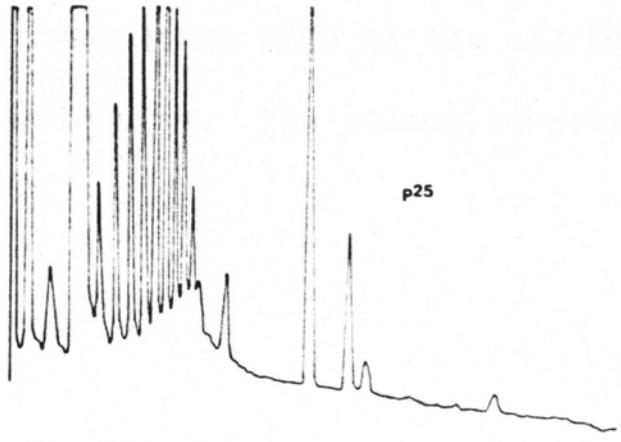

94,000-
67,000-
43,000-
30,000-
20,100-
14,400-
a. b. c.
d.

Figure 7. Tryptic peptide graphs from HPLC of the p40, p25, and p16 antigens. The column temperature was maintained at 60°C.


p40


p25

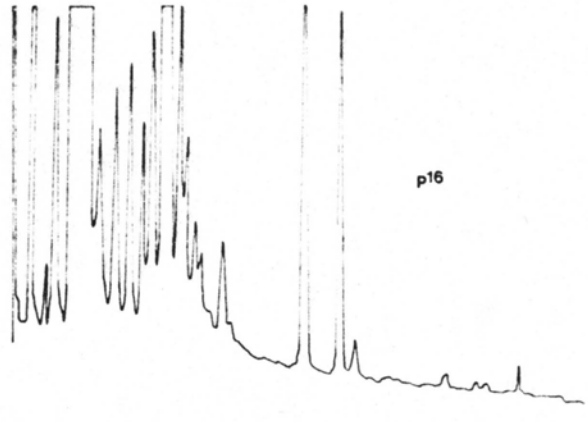

p16

Figure 8. Tryptic peptide graphs from HPLC of the aggregated p25 (a) and the aggregated p40 (b) molecules. The column temperature was maintained at 45°C.

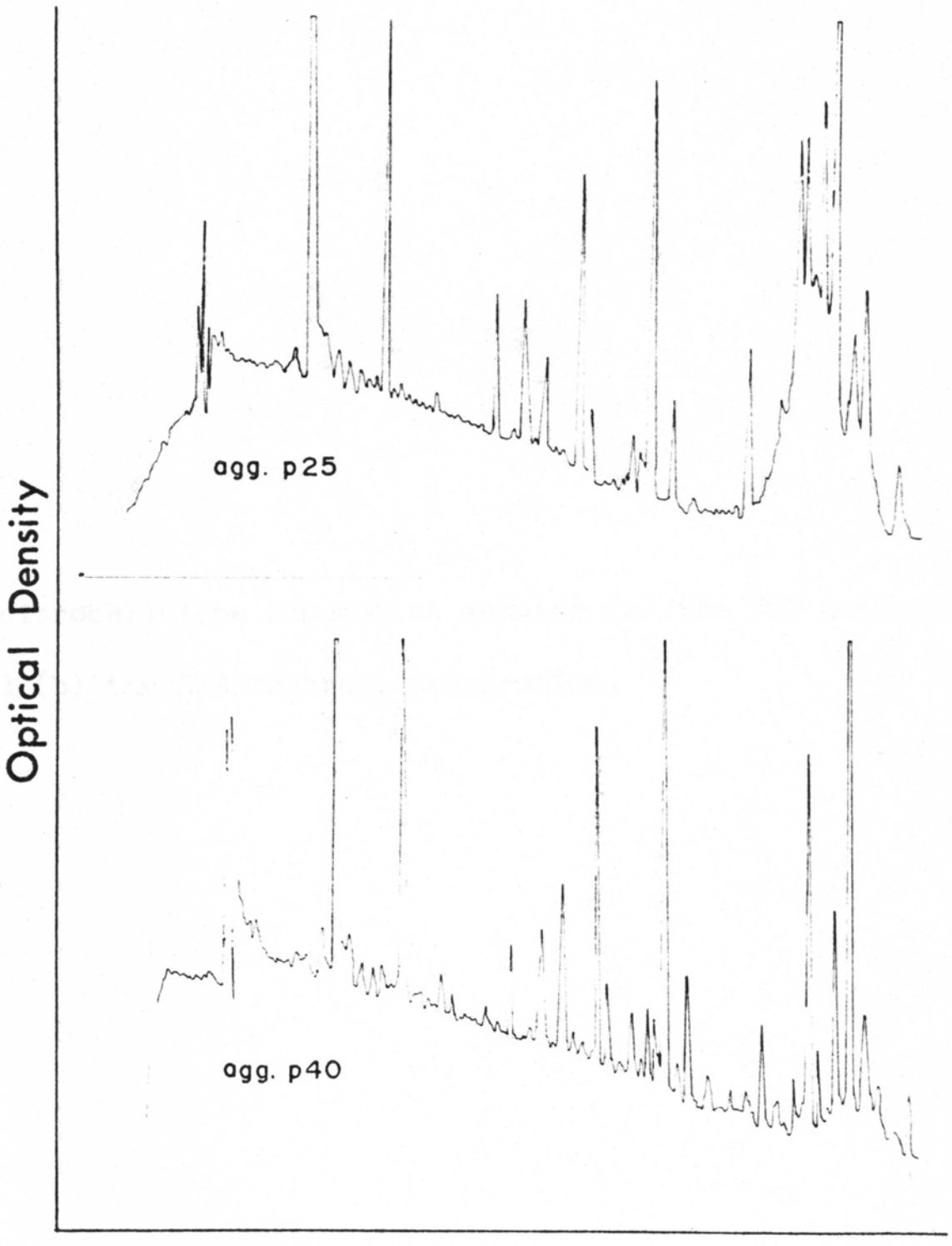
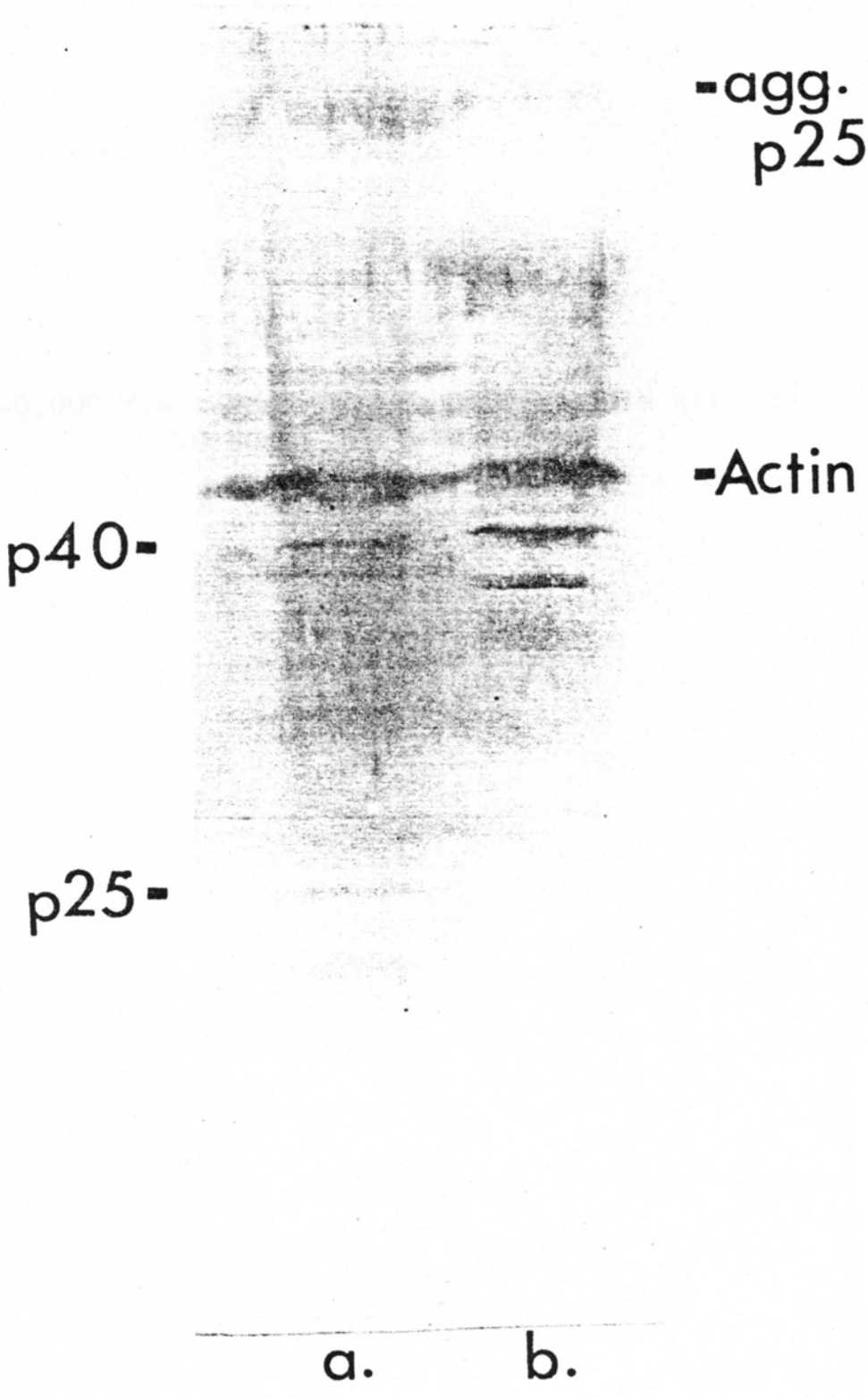

Optical Density
agg. p25
agg. p40
Elution Time

Figure 9. An nitrocellulose immunoblot showing (a) the DOC membrane preparation and (b) the SDS membrane preparation.


-agg.
p25
-Actin
p40-
p25-
a.
b.

CHAPTER 5

A 40,000 M.W. HUMAN THYMUS GLYCOPROTEIN RELATED TO HUMAN THY 1 HOMOLOGUE

SUMMARY

A 40,000 M.W. glycoprotein was isolated from human thymus. This molecule binds lentil lectin, reacts with an antiserum made against the p25 antigen (the human Thy 1 homologue) and possesses almost identical amino acid composition as the p25 antigen and its 40,000 M.W. dimer.

INTRODUCTION

The Theta or Thy 1 molecule was described originally as a murine brain and thymus antigen by Reif and Allen in 1964 (1). It has since served as a cell surface differentiation marker for thymocytes and mature T lymphocytes in mice (2). In rats, Thy 1 appears earlier in development than in mice with the antigen being present, in addition to brain and thymus, on bone marrow (2) and haemopoietic cells (3), but not on peripheral T cells (4-6).

The Thy 1 antigen exists in similar quantities in human brain as compared to rodent brain (7); however, controversy exists concerning the existence of Thy 1 on human thymus (8-10). Dalchau and Fabre did not detect Thy 1 on human thymocytes in 1979 (11), but McKenzie and Fabre did detect Thy 1 in discrete areas of the thymus in 1981 (12), using an antiserum prepared against a human brain Thy 1 antigen. Balch and Ades (13) detected a 25,000 M.W. membrane antigen using an antiserum prepared against monkey thymocytes localized on normal T lymphocytes and human T lymphoblastoid cells. Ritter (14) found human Thy 1 present on 0.1-0.5% of bone marrow cells and 0.2-10.0% of thymocytes, with all Thy 1 positive T cells situated in the outer thymic cortex.

After isolating a 25,000 M.W. glycoprotein from the MOLT-3 human T lymphoblastoid cell line and determining its homology with the murine Thy 1 molecule, we attempted to prove the presence or absence of this glycoprotein on human thymus. Also of importance was the determination of whether the 25,000 M.W. molecule isolated from the lymphoblastoid

cell line was similar if not identical to that found on normal tissue. After using the typical isolation procedure for murine and human Thy 1, little 25,000 M.W. glycoprotein was detected. However, a 40,000 M.W. glycoprotein with characteristics similar if not identical to the human Thy 1 was detected. In a previous study we found that the human Thy 1 or p25 molecule seems to form a dimer of approximately 40,000 M.W. (Bonewald et al., submitted for publication). The 40,000 M.W. molecule isolated from human thymocytes is a glycoprotein, i.e., it binds to lentil lectin, reacts with antisera made against the p25 antigen, and possesses an almost identical amino acid composition as the human Thy 1 and its 40,000 M.W. dimer.

MATERIALS AND METHODS

Normal thymus gland was obtained from patients aged 6 months to 12 years who had portions of this gland removed as a necessary adjunct to cardiac surgery. The thymic fragment was immediately placed in saline, transferred to 1640-RPMI (Gibco), the outer membrane removed, minced by scissors, and pressed through a nylon mesh bag into a single cell suspension. Red blood cells were removed by lysis in a 0.85% ammonium chloride solution and the cells washed in phosphate-buffered saline (PBS) and frozen until enough material was collected for isolation.

Molt-3 cells were grown and harvested as described previously (15,16). Bovine muscle actin was purchased from Sigma.

Isolation procedure

Thymocytes were subjected to one of three isolation procedures: one is the standard procedure for the isolation of rodent Thy 1, one is an abbreviation of this procedure, and one is passage over an immunosorbent column. The standard isolation procedure is shown in Figure 1.

In the second procedure, the material which bound to a lentil lectin column and that which did not bind was subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (16).

The third isolation procedure was identical to the previous procedure to the point of micelle formation of membrane glycoproteins

in the 2.5% DOC supernatant. This material was applied directly to an immunosorbent column prepared using rabbit anti-p25 antisera (17). After application, the column was washed with PBS. The bound antigen was eluted using 1 M acetic acid, after which this material was air concentrated.

Preparation of immunosorbent column. 10 g of Sepharose 4B was activated by cyanogen bromide essentially as according to the method of March et al. (18) and reacted with 5 ml of rabbit anti-p25 antiserum.

Enzyme-linked immunosorbent assay (ELISA). This assay was performed as described previously (19).

Amino acid composition. The thymocyte antigen, the p25 antigen, and actin were analyzed for amino acid composition by standard procedures using a Durrum D-500 automatic amino acid analyzer. Approximately 20-50 ug of protein was hydrolyzed in 6N HCl, 0.1% phenol at 110°C under vacuum for 18 hr.

SDS-PAGE. Analytical SDS-PAGE was performed according to the method of Laemmli (20) using a 12.5% gel on a Hoefer electrophoresis apparatus. The gels were stained by the silver nitrate method (21).

Gel densitometry. Gel scanning was performed using a Joyce-Loeble Microdensitometer 3CS (England) with a 15.8 mm slit and a 446 filter of the film positives made of silver nitrate stained polyacrylamide gels.

RESULTS

When human thymocytes were subjected to the identical isolation procedure as used for the isolation of rodent Thy 1 or human Thy 1, little or no 25,000 M.W. bands were seen in the LMW fraction of the S-200 superfine column. These results can be seen in densitometer tracings of 12.5% SDS-PAGE as shown in Figure 2. The LMW fraction from Molt-3 cells contains a great deal of the 25,000 M.W. or p25, with smaller amounts of bands at the 40,000 and 16,000 M.W. area. The thymocyte fraction showed no major band.

For the next isolation procedure on human thymocytes, the lentil lectin eluted fraction was not applied to the S-200 column. Instead the material which bound to the lentil lectin and the fraction which did not bind to lentil lectin were air dialyzed and subjected to preparative SDS-PAGE. These different fractions were tested in the ELISA assay for reactivity to the anti-p25 antiserum. As shown in Figure 3, only one fraction (fraction #4, corresponding to approximately 40,000 M.W., bound to lentil lectin) reacted with the antiserum; none of the unbound fractions reacted.

Next this bound fraction #4 was reduced and alkylated and reapplied to a preparative gel. A band of approximately 40,000 M.W. still reacted with the antiserum (data not shown).

To decide whether this 40,000 M.W. band was actin, we performed amino acid composition of bovine muscle actin, ran gels comparing migration of the two proteins, and performed an ELISA assay to detect

any reactivity of the p25 antisera with actin. As shown in Figure 4, there is moderate reactivity of actin with the anti-p25 antisera. However, according to SDS-PAGE the actin migrates in the 45,000 to 47,000 M.W. range, and a band corresponding to actin is not detected in the material that we isolated (Fig. 5). Our amino acid compositions of actin correspond very favorably with that reported by Tonomura (22). As shown in Table 1, the amino acid compositions of the human p25 isolated from Molt-3 cells and the 40,000 M.W. protein isolated from human thymocytes differs clearly from that of actin. The key amino acid which demonstrates a distinct difference between the two proteins, actin and Thy 1, is methionine which is of considerably higher amount in actin.

For the third isolation procedure, the human thymocyte 2.5% DOC supernatant was applied to a rabbit anti-p25 immunosorbant column. When samples were taken immediately from the 1M acetic acid elution and electrophoresed on analytical gels, two bands corresponding to approximately 25,000 and 40,000 M.W. were seen and appeared identical to those seen with the same Molt-3 fraction (Fig. 6). However, after this wash had been pooled and concentrated by air dialysis at 4°C over several days and rerun on gels, the lower band had disappeared in the thymocyte concentrate but not in the Molt-3 concentrate (Fig. 7). To determine if the thymocyte 40,000 M.W. band migrated in the same position as the Molt-3 40,000 M.W. band, the further concentrated samples were run simultaneously on a 12.5% SDS-PAGE. Figure 8 shows that the two bands migrate identically.

DISCUSSION

The Thy 1 antigen is probably the most abundant cell-surface molecule found on mouse and rat thymocytes. Reif and Allen (1) found Thy 1 antigen is in greatest amount on murine brain and thymus, followed by lymph node and spleen, but none on bone marrow. Acton and coworkers (4) found the Thy 1 antigen on rat brain and thymus, on spleen and bone marrow, but none on lymph node. Dalchau and Fabre (11) found canine Thy 1 on brain, kidney, thymus, lymph node, spleen, and bone marrow, but found human Thy 1 only on brain and kidney. Although the Thy 1.1 alloantigenic determinant (which differs by a single amino acid substitution from Thy 1.2 in the mouse, arg for 1.1/gln for 1.2) is found in the rat, the 1.2 is not. Neither determinant is shared by canine or human Thy 1 (11).

Arndt and Thiele (23) used anti-human brain antisera absorbed by standard methods and by demonstrating cross-reactivity between species showed little or no reactivity with human thymus. McKenzie and Fabre (12) using an anti-human brain monoclonal antibody showed that human thymus, spleen, and lymph node contain Thy 1 positive components in discrete patches. Saji and Tanigaki's (24) Thy 1-like antigen was found in greatest quantity in brain, followed by testis, but also was found in all lymphoid tissues, including peripheral blood.

Recently, Ritter et al. (14) found human Thy 1 present on 0.2%-10% of thymocytes in addition to bone marrow, 0.1-0.5%. Their data demonstrated that Thy 1 is present on early stages of both T and B lymphocytes. Their antisera included a rabbit anti-rat brain Thy 1 and

seven monoclonal antibodies to human brain Thy 1. Ades and co-workers (13) used an antiserum prepared against monkey thymocytes to identify a 25,000 dalton surface protein on normal human T lymphocytes. Therefore, confusion continues to exist concerning the presence or absence of Thy 1 on human thymocytes and T lymphocytes.

For our detection method, we have used standard isolation procedures for the theta antigen (25,26) and an immunosorbant column made with rabbit anti-p25. The p25 is an antigen purified from a human lymphoblastoid cell line, Molt-3. This replaces the serological immunosorption experiments used by previous investigators.

In a previous paper (Bonewald et al., submitted for publication), we have shown that the human Thy 1 or p25 antigen can be converted to a 40,000 M.W. protein, especially after exposure to SDS. Sauser et al. (27) using SDS for detergent and a solubilization agent of a cell membrane fraction from mouse thymocytes, isolated a 40,000 M.W. Thy 1 antigen. This is in contrast to the 25,000 M.W. Thy 1 isolated using DOC as the solubilizing agent (7,25,26,28).

Only two pieces of our data show that the Thy 1 may exist in a 25,000 M.W. form on human thymocytes. One is the 25,000 M.W. peak seen on gels immediately after the 1 M acetic acid elution of the anti-p25 column. The second is a faint band in the area of 25,000 daltons on the LMW fraction of the S-200 column. Therefore, if the 25,000 M.W. protein is present on the cell surface it is rapidly converted to the 40,000 M.W. form during isolation.

In previous work, we established that SDS could convert the p25 or Thy 1 to a 40,000 form, but from the present data it seems that acetic acid may also cause conversion. The thymocyte molecule may be particularly susceptible to this transformation. Since the amino acid compositions appear similar, perhaps differences lie in the carbohydrate content which could influence susceptibility to conversion. Hoessli et al. (29) determined that T-lymphocyte differentiation in the mouse is accompanied by an increase in Thy 1 sialic acid by neuraminidase determinations. Carlsson and Stigbrand (30) demonstrated carbohydrate complexity in the mouse thymocyte Thy 1 by lectin affinity and isoelectric focusing.

The most pressing question after discovering reactivity of our antiserum with the 40,000 M.W. fraction was -- could we be detecting actin? Actin is a highly conserved protein found in abundance in all eukaryotic cells. The amino acid sequence and composition is highly conserved and it mainly plays a role in muscle contraction. Although actin could be a minor contaminant, our amino acid compositions prove that the thymocyte molecule being detected is not actin. Also, actin migrates considerably slower on SDS-PAGE (45-47,000 M.W. range) than the 40,000 M.W. protein.

Cross-reactivity of our antiserum with actin may be due to contamination of the original p25 preparation used for injection in rabbits. However, the antiserum may be detecting cross-reactivity between the Thy 1 and actin. Dales et al. (31) discovered monoclonal antibodies with affintiy for Thy 1.1 and Thy 1.2 which also bind to

actin and vimentin. Computer analysis of amino acid sequences of murine Thy 1.2, rabbit and chicken skeletal or smooth muscle actin and rabbit vimentin showed suggestive sequence homology. However, exposure of living Thy 1.2 or 1.1 thymocytes to actin antibody did not produce any detectable surface binding which demonstrates the absence of actin from the thymocyte surface.

In conclusion, the human thymocyte Thy 1 must be more similar to the 40,000 M.W. dimer than the 25,000 M.W. antigen isolated from Molt-3, a human T lymphoblastoid cell line.

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Table 1

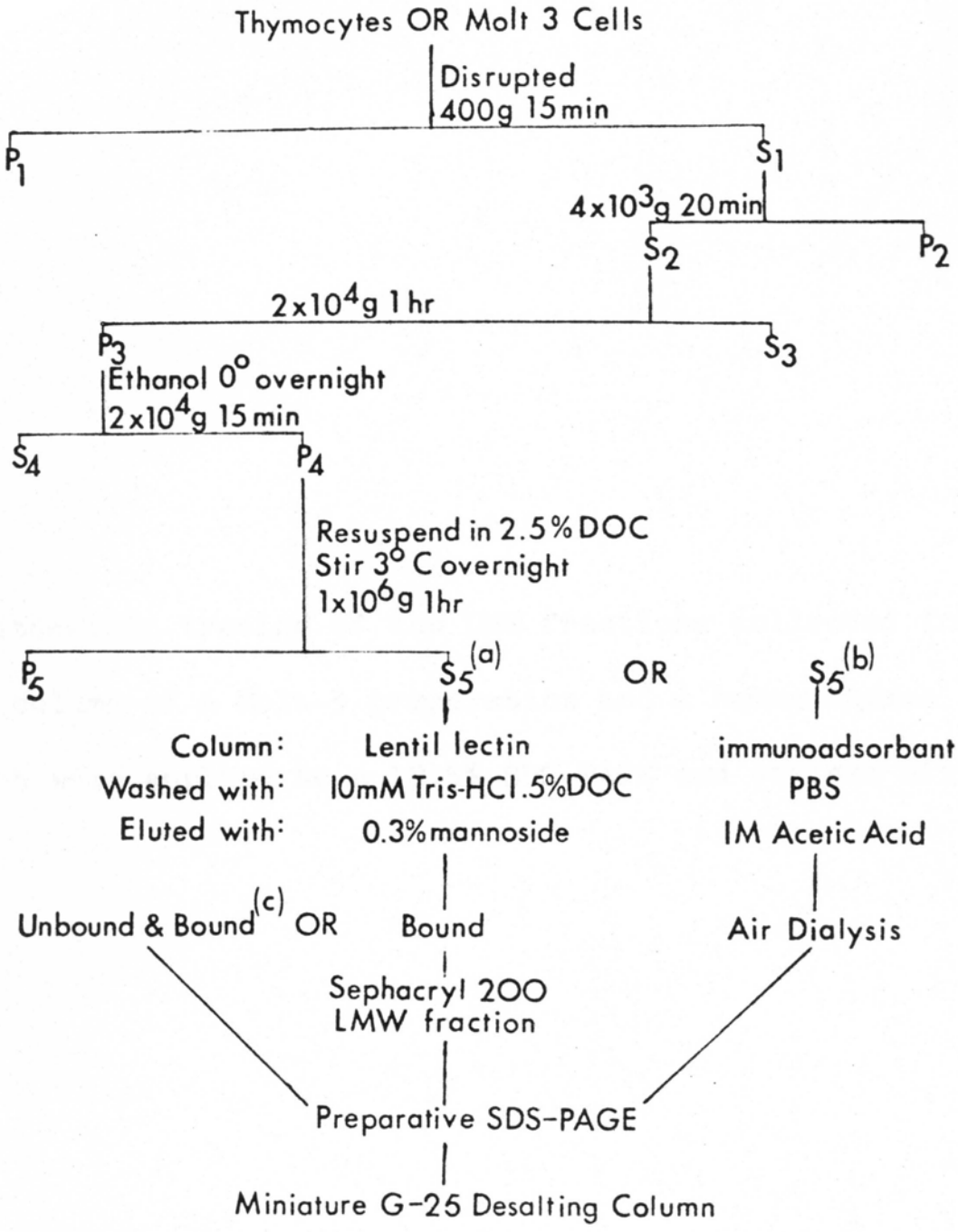
Amino acid compositions are expressed in mol%; tryptophan and cysteine are not included. Data from Tonamura's laboratory was converted from number of amino acid residues for a molecular weight of 4.7×10^4 to mol%.

Amino acid	Actin	Actin*	Human Thy 1	Thymus** antigen
Asp	9.5	9.3	12.0	10.9
Thr	7.5	7.2	6.3	5.1
Ser	6.8	6.4	6.2	6.9
Glu	13.7	10.6	13.4	10.4
Pro	2.2	5.0	5.9	5.5
Gly	10.5	7.4	3.1	5.0
Ala	8.6	8.1	5.7	8.7
Val	4.6	5.2	8.0	6.2
Met	4.7	4.2	1.4	0.7
Ile	6.6	7.5	4.2	3.6
Leu	9.0	7.0	9.6	8.5
Tyr	3.0	4.2	2.6	6.6
Phe	1.0	3.2	3.6	5.1
His	2.6	2.0	1.8	3.5
Lys	5.0	5.6	7.9	5.4
Arg	4.6	5.0	4.8	4.7

*Data from Tonomura et al. (22).

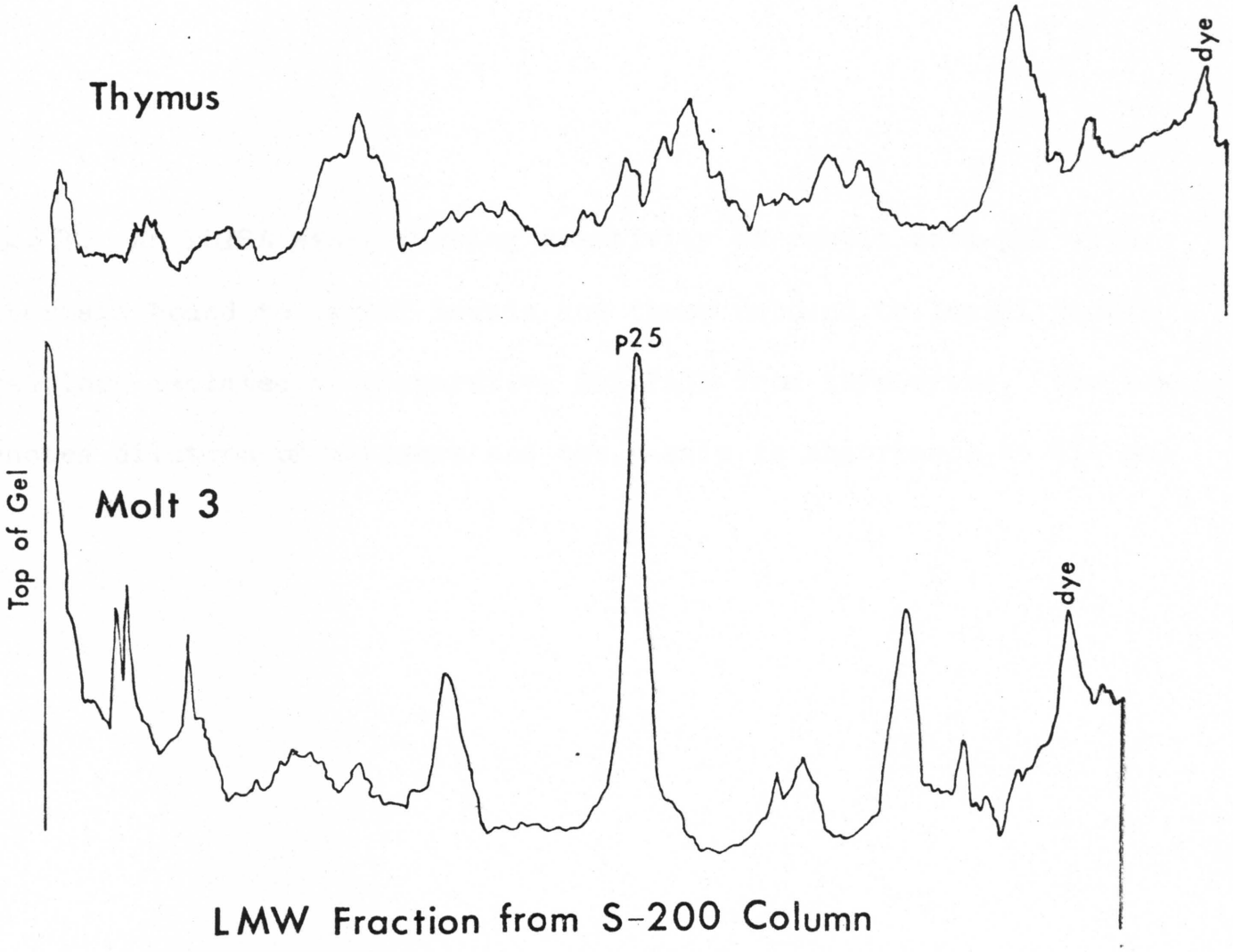
**Corrected values.

Fig. 1. Flow sheet for isolation of human Thy 1 from Molt-3 cells. The same procedures were used in human thymocyte isolation.



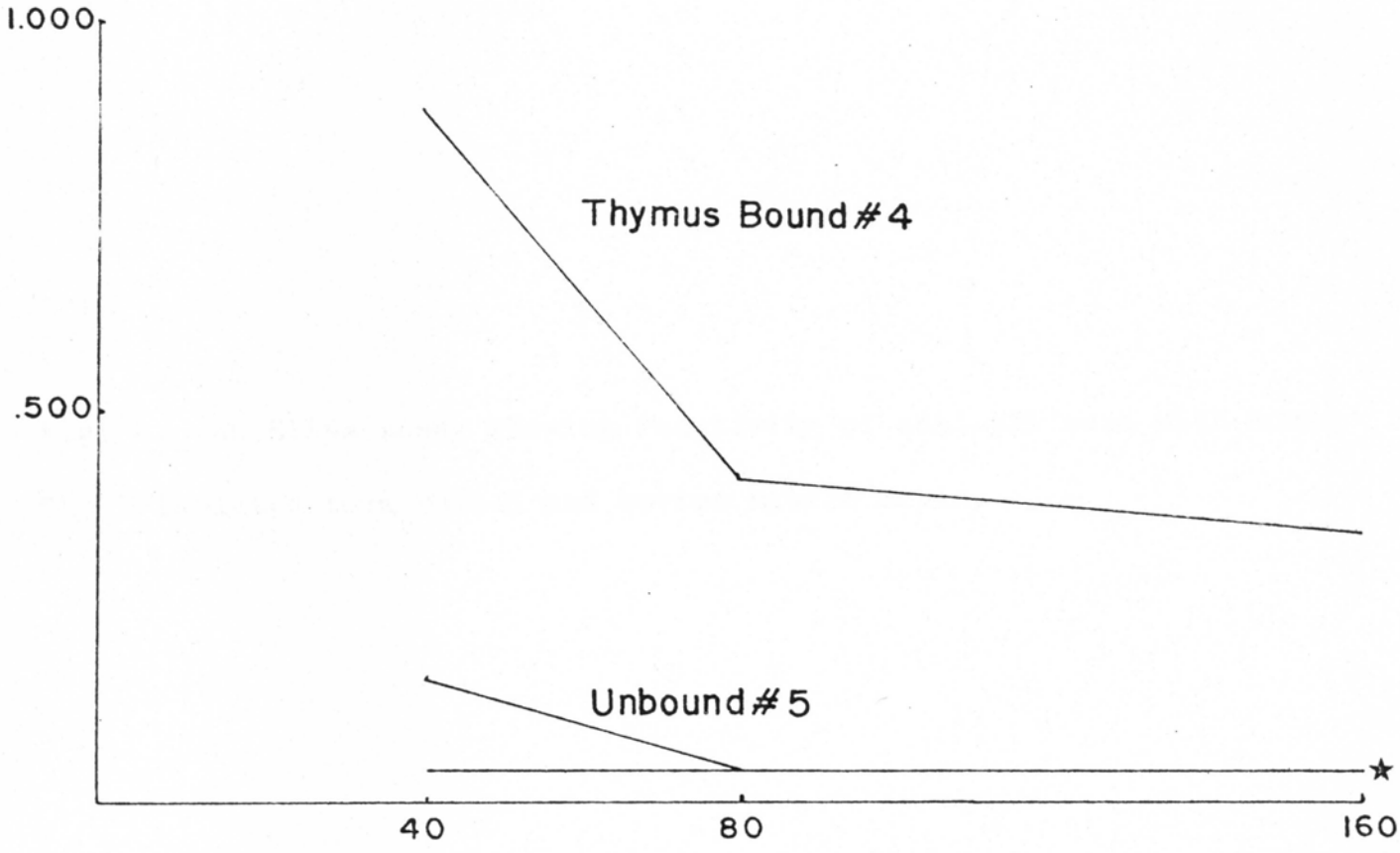
(a) Standard isolation procedure.
(b) Isolation using anti-p25 column.
(c) Fractionation of bound & unbound material by preparative gels.

Fig. 2. A densitometric tracing of the LMW fractions collected from S-200 superfine column of a Molt-3 preparation and a human thymus preparation which were applied to a 12.5% SDS-PAGE and stained with silver nitrate.



LMW Fraction from S-200 Column

Fig. 3. An ELISA assay showing reactivity of rabbit anti-p25 with materials bound to lentil lectin and those unbound to lentil lectin fractions isolated by preparative SDS-PAGE from thymocytes. The x-axis denotes dilution of antisera and the y-axis is absorbance at 414 nm.



★ Includes Bound fractions 1, 2, 3, 5, 6 and Unbound fractions 1, 2, 3, 4, 6, 7

Fig. 4. An Elisa assay showing reactivity of anti-p25 sera with human Thy 1 isolated form Molt-3 and bovine muscle actin.

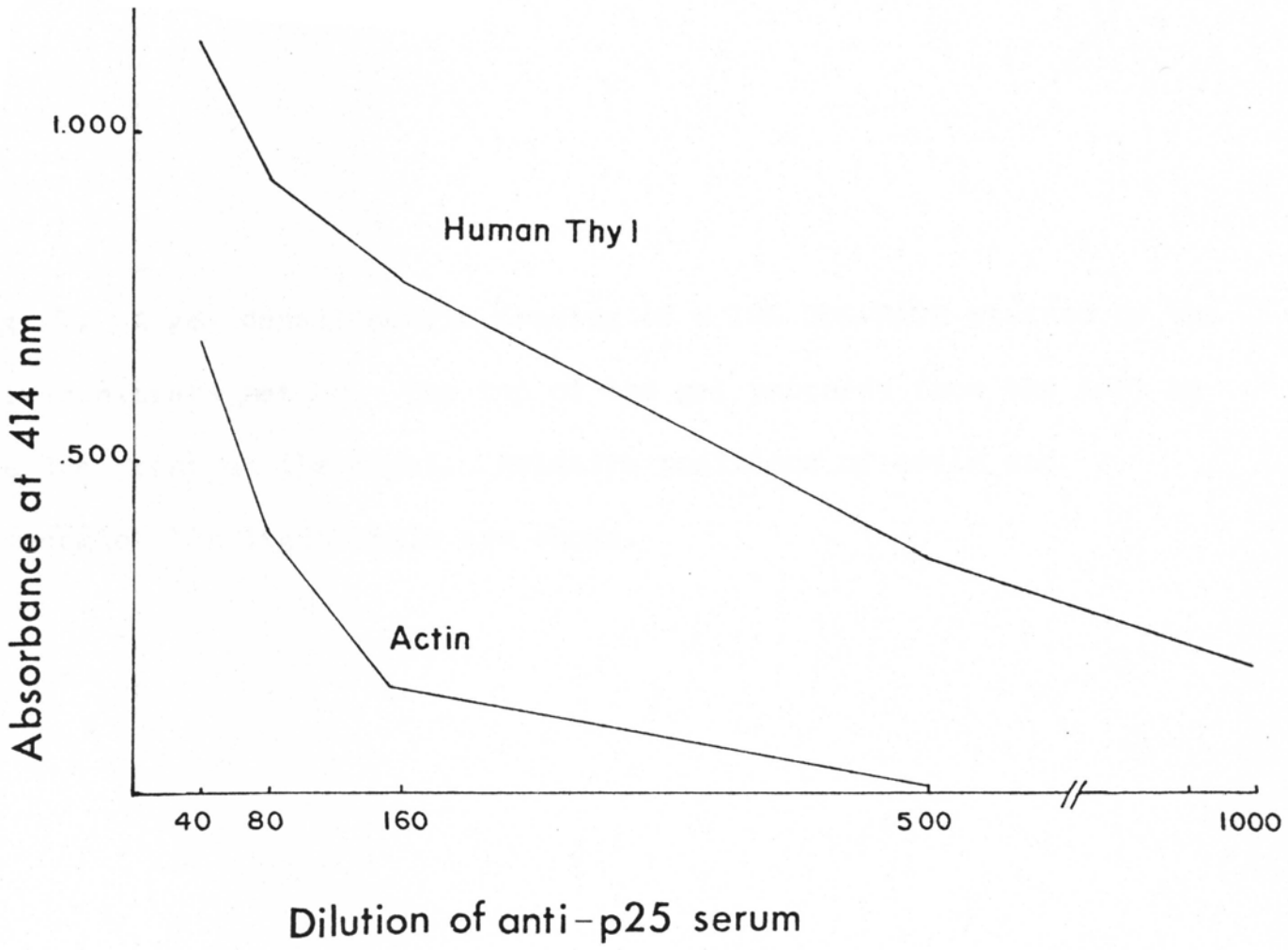
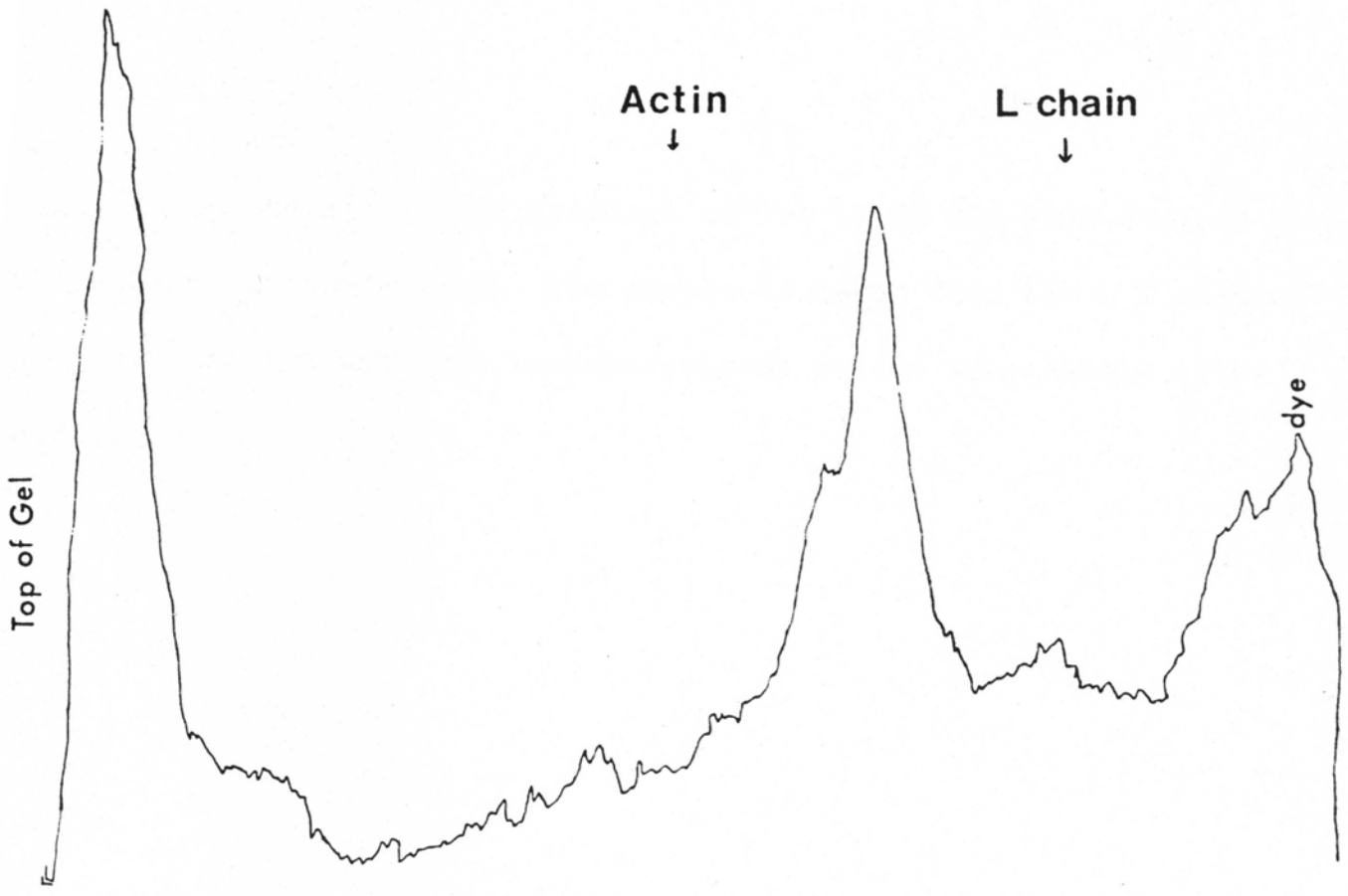
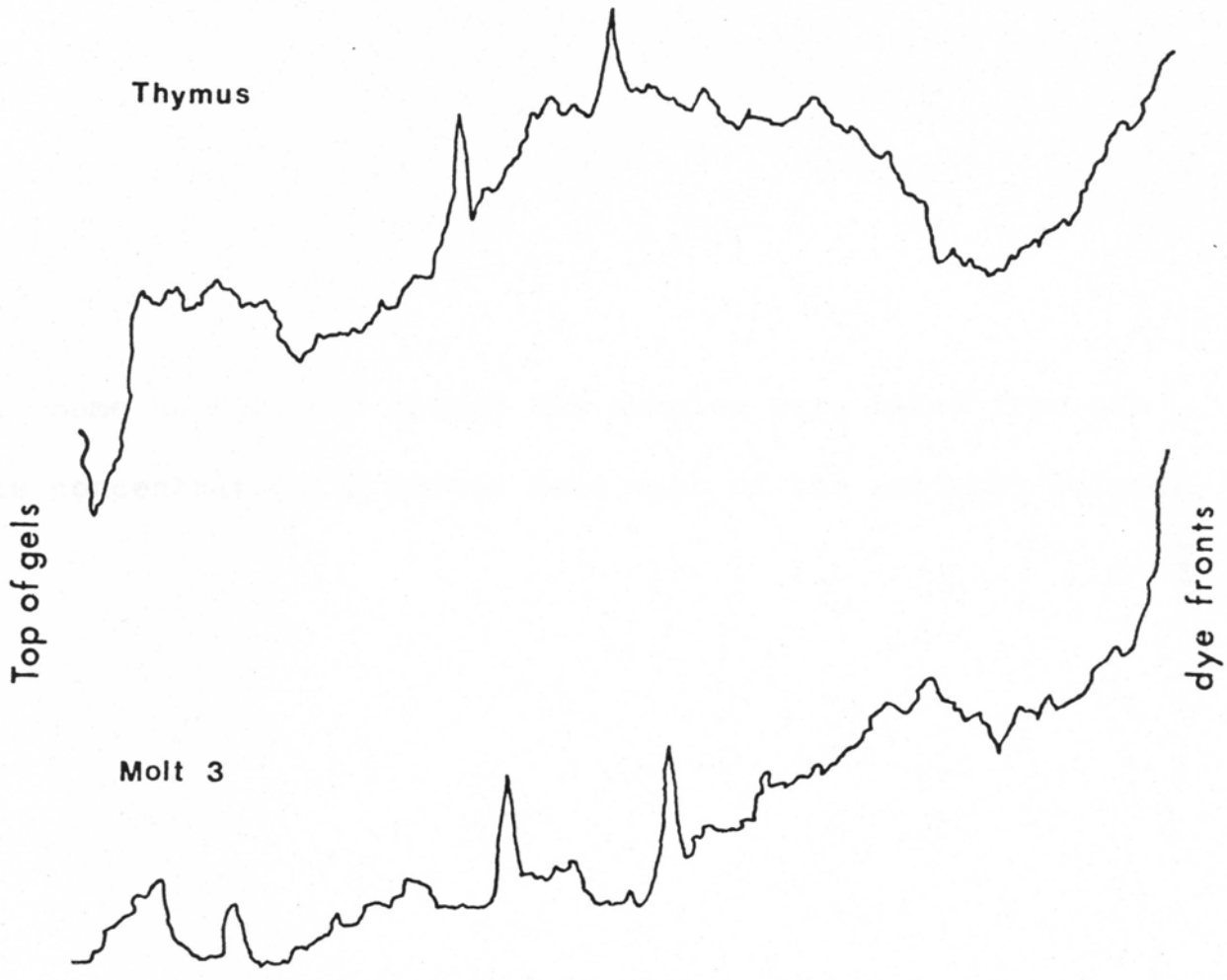

1.000
.500
Absorbance at 414 nm
Human Thy I
Actin
40
80
160
500
1000
Dilution of anti-p25 serum

Fig. 5. A gel densitometric tracing of a 10% SDS-PAGE stained by the silver nitrate method. The top of the gel proceeds from the left to the dye front on the right. Relative positions of actin and immunoglobulin light chain are shown.



Lentil Lectin Bound Fraction #4

Fig. 6. Gel densitometric tracings of two 12.5% SDS-PAGE stained by the silver nitrate method. The sample is taken from the 1 M acetic acid wash of the anti-p25 immunoadsorbent column immediately after elution.



1M Acetic Acid Wash

Fig. 7. Same as Figure 6 except the samples were taken from air dialysis concentrated 1 M acetic acid wash of the anti-p25 column.

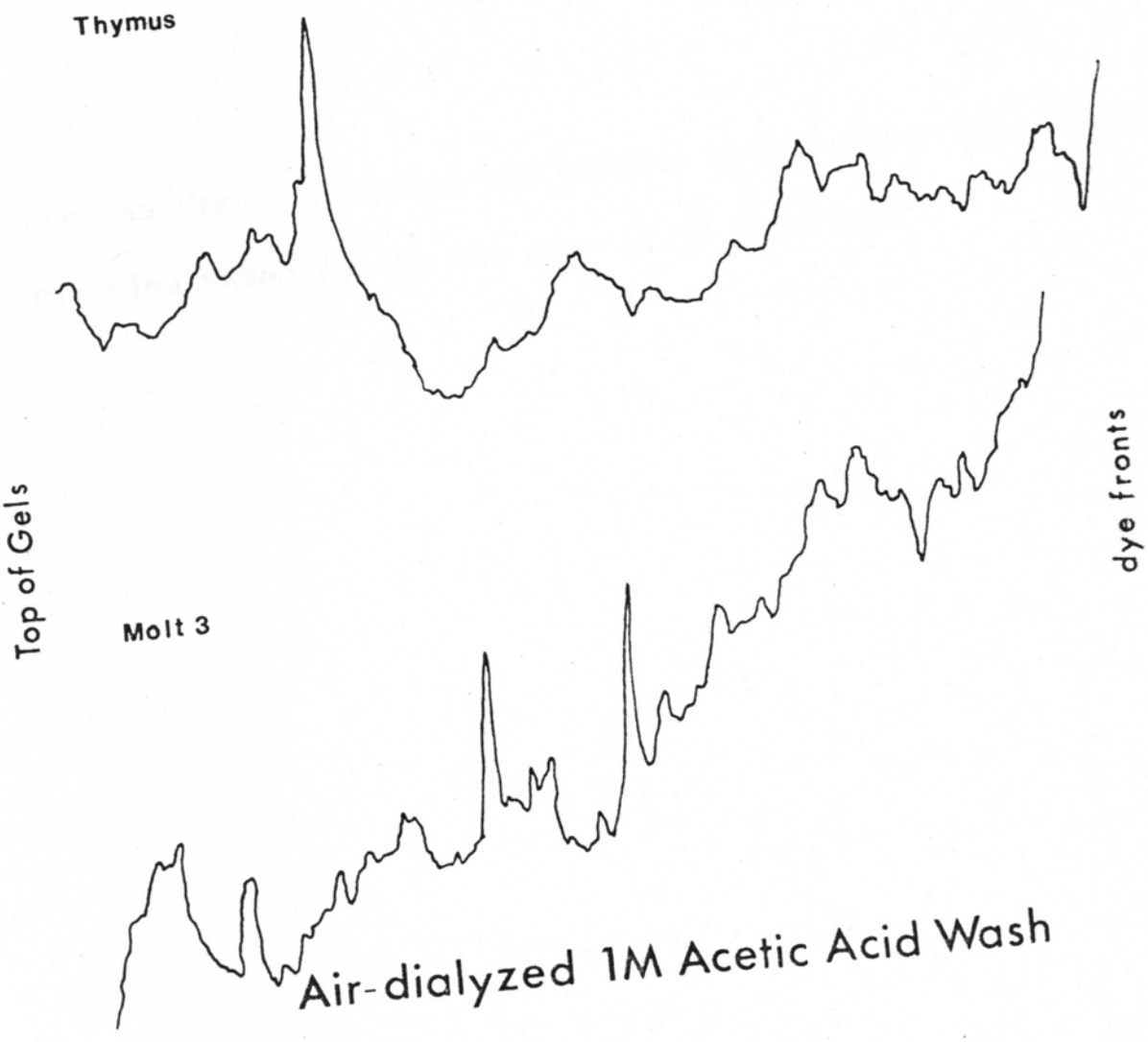
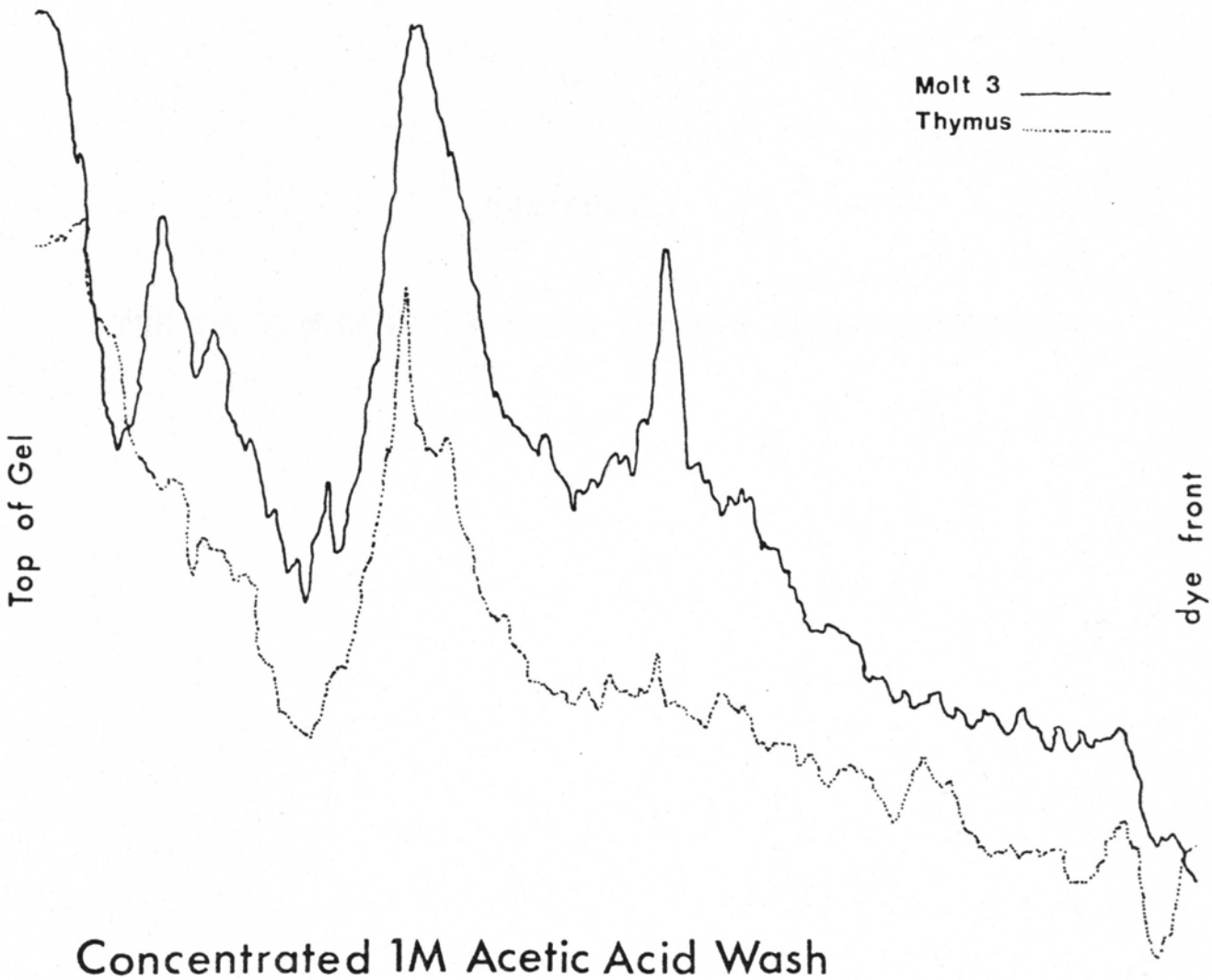

Thymus
Molt 3
Top of Gels
dye fronts
Air-dialyzed 1M Acetic Acid Wash

Fig. 8. Same as Figure 7 except the samples were further concentrated and were run simultaneously on the same 12.5% SDS-PAGE.



Concentrated 1M Acetic Acid Wash

CHAPTER 6

THE THY 1 MOLECULE APPEARS TO BE A LIPOGLYCOPROTEIN

INTRODUCTION

Two other forms of the human Thy 1 (p25) molecule have been noted and biochemically characterized (1). One form is approximately 40,000 MW (p40) according to SDS-PAGE, the dimerized form of p25 and the other is 16,000 MW (p16), possibly a breakdown product of the p25. Both forms are reactive with antisera made to the human Thy 1 or p25 antigen. The p40 and p25 seem to have almost identical amino acid compositions. The amino acid composition of the p16 is also very similar. The carbohydrate composition of the p40 and p25 are very similar while the p16 seems to have little carbohydrate. The most unusual characteristic of these molecules is the possession of lipid components, two fatty acids, again in almost identical proportions in the p40 and p25, yet different although present in the p16. These data seem to suggest that the human Thy 1 is a lipoglycoprotein.

MATERIALS AND METHODS

The p25, p40 and p16 molecules were isolated and purified as described previously using preparative SDS-PAGE and a miniature desalting G-25 column (1).

Fatty acid analysis: The n-hexane extracted material from the hydrolyzed sample from alditol acetate preparations (2) or from Trimethyl silylated (TMS) preparations (3) were evaporated under nitrogen. Approximately 25 ul of Tri-Sil (Pierce) was added to the alditol acetate samples. Normal fatty acids were determined qualitatively and quantitatively by gas-liquid chromatography of the fatty acid methyl esters obtained from the methanolyzate.

The sample was reconstituted in chloroform and injected onto a 10% SP-2330, 6 ft x 2 mm glass gas chromatography column. Initial temperature was 160°C, held for 10 min and increased to 190°C at a rate of 3°/min and held at the final temperature for 20 min. Injection temperature was 250°C. The carrier gas was nitrogen at a flow rate of 20 ml/min. Using a flame-ionization gas chromatograph, 1 ug of each fatty acid could be adequately detected at 32 x 10 -1- AFS sensitivity. The area of the C16 peak was divided by the area of the C18 peak to determine ratios for each molecule tested.

RESULTS

Whether the samples were desalted or not desalted, the same results were obtained on the fatty acid analysis. Table 1 gives the ratio of the first peak to the second peak. The two fatty acids seem to be be C16 0 and C18 1 according to the elution times of the fatty acid standard (Pierce). For three different extractions the ratio for the p25 and p40 were very similar (.68). The ratio for the p16 was always less (.51). Other bands that were eluted from the gel, such as a 12,000 MW,a 20,000 MW and a 67,000 MW bands did not have any fatty acids according to this assay. These two peaks were not present when the aggregated p25 and p40 were analyzed. In one assay more than two peaks were seen.

DISCUSSION

There is an unusual carboxyl terminal found on the Thy 1 molecule. When sequencing rat brain Thy 1, Campbell (4) could obtain the C-terminal peptide either in highly aggregated form or could purify it only in Brig 96 detergent. It appeared to have hydrophobic properties yet contained few hydrophobic amino acids. He found unidentified ninhydrin-positive material and glucosamine and galactosamine. He suggested that the C-terminal may be linked to lipid. If so, this would make the Thy 1 and unusual molecule indeed. Barclay and coworkers (5) mentioned minor unidentified peaks in carbohydrate analysis which they thought to be stearic acid. Other investigators such as Sauser (6) assumed a molecular complex between a protein moiety and a glycolipid existed for the Thy 1. If lipid is present, is it covalently or hydrogen bonded to the molecule?

We performed a fatty acid analysis on the n-hexane extraction of hydrolyzed p40, p25, or p16. Surprisingly, we found two fairly common fatty acids. The ratio of the C16 to the C18 fatty acid was almost always identical in the p40 and p25 molecules, however the p16 ratio was always less. Again this demonstrates that the p40 and p25 are the same molecule and the p16 is differing in the aspect of lipid content in addition to possessing less carbohydrate (1). We assume that these two fatty acids are part of a lipid moiety bound to the molecule. The lipid moiety must be covalently bound since the antigens were extracted from a Tris-glycine SDS-polyacrylamide gel. Normally free lipid components would migrate to the dye front. This would also explain our loss of the p25 antigen when dialyzed against buffer containing no

detergent. Sauser (6) also found that prolonged dialysis against cold distilled water or repeated freezing and thawing of the protein reduced antigenic activity. Lipid has a high affinity for dialysis membrane.

There are very few examples in the literature of lipid covalently attached to protein. One example is that of Hantke and Braun (7) who characterized a murine-lipoprotein, in which a thioether bond linked a cysteine side chain to a diglyceride and a third fatty acid is attached to the N-terminal amino acid group of the cysteine. Fatty acids can also be covalently attached to carbohydrate such as the sugar glucose in the case of acyl-glucosylceramides (8). We tend to favor the proposal that lipid is attached to the p25 molecule via a carboxyl terminal cysteine similar or identical to cysteine #111 or #112 identified from sequence data of mouse and rat Thy 1 (4,9).

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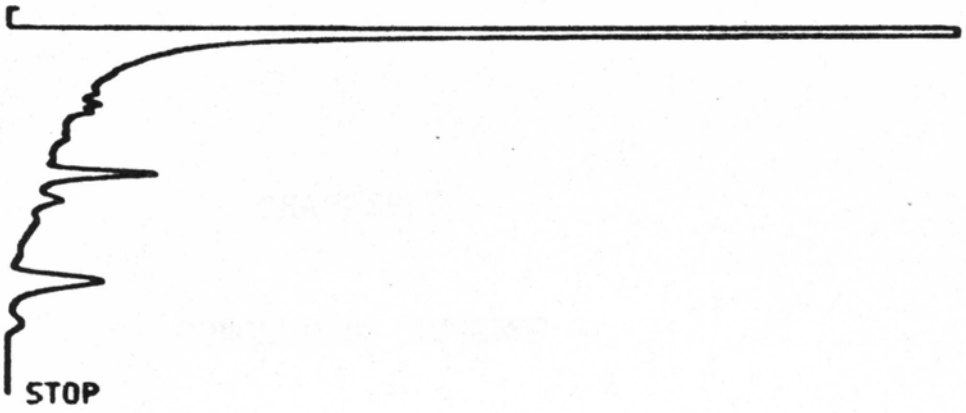
Table 1. FATTY ACID ANALYSIS

ratio of $\frac{C_{16} \text{ peak}}{C_{18} \text{ peak}}$	p25 = .68 (3) p40 = .68 (3) p16 = .51 (3)
% composition of p25	2.7% C_{16_0}
	3.1% C_{18_1}
	6.8% total

The number in parenthesis is the total number of extractions tested.

Figure 1. A gas chromatograph tracing of a fatty acid analysis of the p25 molecule showing the presence of two peaks corresponding to C_{16} and $C18_1$.

START 00.00.00.00.



C-R1A
SMPL # 00
FILE # 8
REPT # 247
METHOD 344

#	NAME	TIME	CONC	MK	AREA
		2.18		V	18200
		2.52			42131
		2.69		V	42657
		3.41			14732
1		3.9	0		9075
		4.32			409752
		4.98			88964
		5.49			41952
		7.1			594015
		8.18		E	80650
	TOTAL		0		1342130

CHAPTER 7

CONCLUDING REMARKS

The Thy 1 or theta antigen was one of the first differentiation antigens on lymphocytes to be described. However useful it has been in the mouse model, to date such does not hold true for the rat, dog, or human since the antigen does not show similarity in tissue distribution across species except for expression in brain. When this study began few human lymphocyte antigens had been biochemically characterized beyond molecular weight determination. This was due to two reasons: one, the unavailability of human tissue and, two, the relatively low density of membrane antigens on cell surface making necessary large amounts of material or tissue in order to isolate purified antigens in sufficient amount for biochemical studies. In our case, this problem was overcome by utilizing large-scale cell cultures of a human T lymphoblastoid cell line, Molt-3 (1). The same isolation procedure using DOC as solubilizing detergent as used for most murine Thy 1 isolation was used for human material. A T cell antigen of molecular weight 25,000 was isolated from the Molt 3 cell line (2).

The p25 antigen when compared to the murine Thy 1 had the same molecular weight, bound to lentil lectin (a characteristic of glycoproteins which possess mannose), and by using immunodepletion and immunoabsorption experiments it was revealed murine Thy 1.2 and p25 were cross-reactive. This immunological data was borne out by biochemical data showing similarity but not identity utilizing amino acid composition, tryptic peptide maps and carbohydrate analysis. In addition, our data demonstrated antigenic determinants shared by murine IgG and human Thy 1 as well as human thymocyte. We also demonstrate a dimeric form and a non-carbohydrate bearing form of the molecule. It

is our contention that the p25 antigen is the mouse Thy 1 or theta equivalent.

Antiserum made to the isolated and purified T cell product, the p25 molecule, reacted strongly with a purified B cell product, human IgG. Cross-reactivity with human IgM, IgA, primate Ig, murine Ig, and a battery of other antigens is low or non-existent. Both the Fab and Fc portion of the human IgG molecule reacted with the antiserum, wherease reduced and alkylated, or trypsin-digested IgG did not. This suggests a defined, tertiary structure of the molecule which includes the disulfide bonds as necessary for reactivity. Carbohydrate moieties on the IgG molecule were definitely ruled out while the disulfide bonds of the first and third domains of human IgG are implicated as the reactive site. Perhaps, isolation and purification of the p25 molecule using DOC exposed new antigenic sites not exposed on the cell surface, thus the cross-reactivity to IgG. Molt 3 does not possess nor synthesize IgG.

These observations are interesting in light of theory recently proposed by Williams and Gagnon (3) suggesting that the Thy 1 molecule may be or is like the primordial immunoglobulin domain or the theory proposed by Jensensius and Williams (4) that the Thy 1 molecule is the T cell receptor. Our data seems to support the previous proposal of homology between Thy 1 and Ig, however, the latter proposal remains as speculation.

Recently, utilizing monoclonal antibodies there have been examples of shared antigenicity of Thy 1 and actin (5) and Thy 1 and a component

of intermidate filaments (6). These investigators also suggest homology based on a computer search and comparison of sequence data. Extrapolating from shared antigenic determinants to molecular homology, and especially extrapolating further into shared similar function is a tenuous area of speculation.

During isolation of the p25 antigen, two other forms of the antigen were noted and characterized. One being the dimerized p25, is designated the p40 molecule. At first appearance, this band on SDS-PAGE was confusing until it was noted that preparative SDS-PAGE greatly increased its presence. Immunoblot experiments of DOC membrane extractions and SDS membrane extraction showed that SDS converts the p25 form into the p40 form. This supports Sauser et al. (7) whose data in 1974 demonstrated that the mouse Thy 1 is a 40,000 dalton molecule. Sauser is the only investigator in the literature who used 2% SDS instead of 2.5% DOC to isolate the molecule. (Brig 96 and Nonidet have also been used.) Other investigators (8) have assumed that a contaminating molecule of 40,000 daltons was present in Sauser's thymocyte preparation and assume a "true" molecular weight of the Thy 1 molecule to be 25,000. Our data are the first to support and vindicate Sauser's work.

The other form isolated was the p16 molecule which is possibly a breakdown product of the p25. Ades and coworkers (14) found a 16,000 MW molecule associated with suppressor cells. The Molt 3 cell line possesses both the p25 and the p16, whereas the Molt 4 possesses the p25, yet lacks the p16 antigen. Although biochemical studies bore out

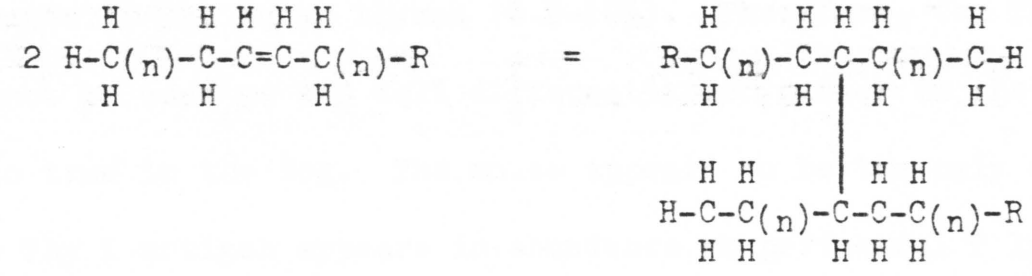
the identity of the p40 and p25 molecule, the same was not true for the p16. Amino acid compositions were very similar which suggests the protein structure or backbone was intact. However, the p16 appeared to possess considerably less carbohydrate than the p25 molecule, yet retained lipid material according to fatty acid analysis. (Such a molecule has not been reported in the murine system). The p16 could be a cryptic antigen, i.e., part of the p25 structure removed by enzymatic degradation. Perhaps the p16 could be the p25 processed in the cytoplasm without the attachment of carbohydrate and then inserted in the membrane. The p16 molecule may play a role in suppressor T cell function or be a differentiation antigen co-expressed on these cells.

Determination of molecular weight is dependent on the method of isolation used and this statement is especially true when dealing with surface membrane antigens. Membrane molelcules are usually present in relatively small amounts and detergent is necessary for their isolation. Detergent can be difficult to work with and can interfere with biochemical methods such as amino acid sequencing. The histocompatibility leukocyte antigens (HLA), though membrane antigens, were sequenced fairly easily because it was determined that a papain single enzymatic cleavage site close to the cell surface would separate the majority of the molecule from its hydrophobic tail piece (9,10).

The standard isolation procedure used for isolation of Thy 1 utilizes DOC and yields a product of 25,000 MW by SDS-PAGE. However, by sedimentation studies the MW is 17-18,000 (11). We have shown that by using SDS in place of DOC the molecule's apparent molecular weight

is 40,000. Atwell and Marchalonis (12) isolated a 60,000 MW theta antigen. This could be due to further aggregation of the molecule since they used an immunoabsorbant column in their isolation proceudre and eluted the material with 1M acetic acid. This is in contrast to Cotmore (13) who also used an immunoabsorbant column, but eluted with diethylamine and retrieved a molecule of 25,000 MW. In our studies, when the p25 and p40 were fixed in a TCA-Coomassie blue solution, they both aggregated to approximately 110,000 MW. Kuchel (11) found mouse Thy 1 aggregates in the absence of detergent to a molecular weight of 230,000-300,000. Therefore, consideration of isolation procedures is important in reporting molecular weight.

The p40 or dimerized form of the p25 antigen is a curious molecule because it does not revert back to the p25 form when treated with a reducing agent. This suggests that dimerization is not due to disulphide bond interchange. Perhaps another form of interchange has occurred and we speculate that the hydrophobic portion of the molecule plays a role in this. If lipid or fatty acids are present, any unsaturated bonds may interchange between fatty acids.



Of course at his time, this speculation remains to be proved.

It was of importance to determine whether the p25 molecule could also be found on normal tissue and whether identity existed with the

p25 material isolated from the lymphoblastoid cell line. After using similar amounts of human thymocyte as used in a Molt 3 isolation, little if any 25,000 MW band was seen on SDS-PAGE of the LMW peak from gel filtration. Yet when preparative gels were run on thymocyte material which was concentrated from the bound and unbound eluate of a lentil lectin column, a fraction corresponding to bound, 40,000 MW, reacted with anti-p25 serum. None was noted in the 25,000 MW range. When a DOC membrane preparation of human thymus was applied to an anti-p25 immunosorbant column, washed and eluted with 1 M Acetic Acid, two peaks were observed on SDS-PAGE. When concentrated by air dialysis only a single band was observed on gels. Some conversion had occurred in the Molt 3 preparation but not to the extent of complete conversion as seen with the thymus material. The thymus material and p25 antigen had very similar amino acid compositions.

The antigen may exist in the 25,000 MW form on the human thymocyte yet because it is present in quantities considerably less than that found on Molt 3 (<10%), perhaps it is more readily converted into a dimerized form. Ritter (15) also found Thy 1 to be present in small amounts in the human thymus (0.2-10%). Therefore, the Thy 1 antigen cannot be used as a T cell differentiation marker in the human, as is also true in the dog. The mouse appears to be the only animal in which the Thy 1 antigen appears in abundance on peripheral T lymphocytes. Only in rodents does the Thy 1 appear in abundance on thymus, in contrast to the dog and human.

Our studies suggest the presence of lipid in the p25 molecule. This is shown by 1) the necessity of detergent for its isolation, 2) the removal of detergent by dialysis results in loss of the p25 molecule and 3) n-hexane extractable material in hydrolyzates of the p25 molecule. The material which lends hydrophobicity to the molecule is probably covalently attached.

In summary, evidence which supports the homology of murine Thy 1 and the human Thy 1 or p25 antigen is:

1. both are 25,000 MW by SDS-PAGE.
2. both are glycoproteins which contain high mannose content, i.e., they bind to Lens culinaris lentil lectin.
3. Cross-reactivity has been shown by immunoabsorption and immunodepletion experiments.
4. Amino acid compositions are similar, yet not identical.
5. Tryptic peptide maps are similar, yet not identical.
6. A 40,000 MW form can be seen with SDS-conversion.
7. HPLC tryptic peptide maps of the p25 contain 12-14 peptides as would be expected for the number of arginines and lysines in the amino acid compositions.
8. The p25 molecule possesses approximately 20% carbohydrate compared to the murine content of 20-30%.
9. The N-terminal amino acid of the p25 appears to be blocked as is true of the rodent Thy 1. Serine appears to be the amino acid following methionine as is true of mouse but not rat Thy 1.

Additional observations accomplished include; antigenic determinants shared by human IgG and human Thy 1 antigen, the presence of a molecule related to the p25 of 16,000 MW, and a human thymus 40,000 MW glycoprotein is related to the human Thy 1.

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Appendix

Distribution of Cell Surface Thy 1 Antigen in Human Haemopoietic Cell Lines as Compared to Balb c Thymocytes and a Primate Cell Line.

Cell Line	Binding Counts	100% Lysis	Neg. Control	Dilution (1:X) % Lysis				
				X=4	8	16	32	64
T Lymphoid Lineage								
Molt 3*	2,000	70	15	23	-	-	-	-
Molt 4*	5,000	71	17	27	-	-	-	-
Molt 21*	7,000	76	20	-	-	-	-	-
HPB-MLT*	2,000	84	33	48	41	-	-	-
HPB-ALL*	2,000	72	23	-	-	-	-	-
JM*	2,000	81	21	-	-	-	-	-
SB	2,000	57	17	-	-	-	-	-
W.M. (woolly monkey T cell)	6,000	85	13	-	-	-	-	-
Balb c thymocytes	300	89	53	80	84	84	79	63
CEM band				+	+	-	-	-
CEM pellet				-	-	-	-	-
B Lymphoid Lineage								
Raji	3,000	79	12	78	69	29	15	12
Nalm 1**	6,000	82	18	42	27	22	-	-
Nalm 6**	600	73	46	51	-	-	-	-
PC 115	15,000	66	20	45	28	12	-	-
RN 114	3,000	82	12	-	-	-	-	-
RPMI 8392	11,000	46	13	-	-	-	-	-
Ball 1	4,000	83	13	-	-	-	-	-

*Early T cell
**Pre B cell

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